UNIQUE PHENOTYPIC AND FUNCTIONAL PROPERTIES OF A CANCER VACCINE BASED ON ATTEUNATED *LISTERIA MONOCYTOGENES*

by
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ABSTRACT

Live, attenuated vaccinations have been successful in the generation of protective immunity to a variety of illnesses, such as the measles, mumps, and rubella, but have had limited success as therapeutic cancer treatments. We explored the differences in the cellular and molecular immunity generated by a new *Listeria Monocytogenes* vaccine platform, as compared to the well characterized Vaccinia Virus. *Listeria*-OVA vaccination resulted in the slowest tumor growth in subcutaneous models, as well as decreased tumor burden and increased survival in a metastatic model. This benefit was dependent on the generation of adaptive immunity, as antibody depletion of $CD8^+$ T cells significantly impaired the survival benefit of the *Listeria*-OVA vaccine. To examine the effects of these vaccines on the early stages of $CD8^+$ T cell development, we adoptively transferred OVA specific $CD8^+$ T cells (OT-1) into host mice prior to vaccination. Surprisingly, we found that *Listeria*-OVA vaccine resulted in $CD8^+$ T cell activation without significant expression of PD-1 or LAG-3, unlike the Vaccinia-OVA vaccine or any other vaccine currently reported. The activation of $CD8^+$ T cells without PD-1 was completely dependent on the genetic deletion of the ActA gene from the *Listeria* vector, as wildtype *Listeria*-OVA did not replicate this phenotype. Strikingly, despite differences in wildtype mice, both vaccines were similarly effective in increasing overall survival in $PD\text{-}1^{-/-}$ mice, suggesting that the difference in induced PD-1 expression was responsible for the difference in efficacy. To better understand this phenotype, we developed a direct *ex vivo* antigen detection assay, and found that both Vaccinia-OVA and *Listeria*-OVA are primarily presented to naïve $CD8^+$ T cells by $CD8\alpha^+$ dendritic cells ($CD8\alpha^+$ DC), and that *Listeria*-OVA vaccinated $CD8\alpha^+$ DCs are sufficient to drive naïve $CD8^+$ T cell

division without PD-1 expression in a contact dependent manner. Finally, we performed microarray analysis on $CD8\alpha^+$ DCs 24 hours after vaccination, and found a dramatic difference in the RNA expression profile of a *Listeria*-OVA vaccinated $CD8\alpha^+$ DCs. Together, these data show that checkpoint protein expression is not uniformly associated with $CD8^+$ T cell activation, and that individual cancer vaccine vectors may increased clinical benefit due to their modulation of checkpoint protein expression.

Thesis Readers

Charles G. Drake, MD, PhD, Thesis Advisor

Leo Luznik, MD

PREFACE

The following work was done in an attempt to further my personal knowledge of the field of immunology, as well as to try and advance the understanding of the field as a whole. Doing these experiments has helped me to realize that there will always be new questions to answer, and that it is as important to enjoy the process of discovering new ideas and information as it is to enjoy the final product.

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passion for the field of study to complete this degree. However, many days of failed experiments ended with the same question, “What am I even doing here?” My family was always supportive, and reminded me that pursuing scientific research was something I love to do, even when it is frustrating.

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CHAPTER I

INTRODUCTION

Introduction

In the past two decades, the field of immunology has begun to develop an understanding of the complex interactions between the immune system and cancer. From the findings that immunocompromised mice[1] and humans[2,3] have an increased incidence of tumor formation, to the discovery that cancer patients have tumor specific $CD4^+$ and $CD8^+$ T cell populations, it has become clear that the immune system plays a prominent role in the control of cancer. Yet, by the time of clinical diagnosis, the tumor has been edited to avoid immune surveillance, as was shown in an elegant series of animal studies. In these experiments, it was found that tumors that had been grown in the absence of an adaptive immune system were often rejected or slowed when transferred into an immune-competent host[4]. However, when the tumors were grown in an immune-competent host first, growth was no longer impeded when they were transferred to a new host. Together, these data suggested that the immune system is capable of rejecting cancer, and does interact and recognize cancerous cells naturally. Yet, by the time of clinical treatment, the tumor has already avoided or tolerized the immune system. Therefore, the goal of cancer immunotherapy is to reactivate the immune system, and provide acute and long term rejection of cancer in the form of immune memory. Two areas of preclinical research that have shown the most progress are the development of cancer vaccinations and the blockade of checkpoint proteins with monoclonal antibodies.

$CD8^+$ T Cell Checkpoint Proteins

Since the arrangement of the T Cell Receptor (TCR) is random, it is unavoidable that some $CD8^+$ T cells will be generated with a TCR that is specific for a peptide that the

host naturally expresses. Due to the sterilizing nature of immunity, the generation of an immune response to a host protein can result in "horror autotoxicus," or autoimmunity. Such a response can be seen in the case of type I diabetes, where patients have $CD8^+$ T cells that have targeted the β–islet cells of their liver, resulting in the lifelong eradication of these cells and the subsequent lack of insulin production capabilities. To prevent this, in addition to central tolerance and $CD4^+$ regulatory T cells, there are a series of extracellular receptors called checkpoint proteins that are imposed on the $CD8^+$ T cells when they are activated in an inappropriate context[5]. These proteins have a role in the prevention of autoimmunity, but also have been found to be used by tumors as a mechanism of immune escape. Many tumors express the ligands for these checkpoint proteins, and tumor infiltrating lymphocytes are often found expressing one or more of these negative regulators of the immune system. This makes checkpoint proteins an attractive target for the generation of anti-tumor immunity[5], though future research will need to continue to explore the signaling pathways by which these proteins act.

Cytotoxic Lymphocyte Antigen-4

The most well understood of the checkpoint proteins is called Cytotoxic Lymphocyte Antigen-4, or CTLA-4. CTLA-4 is a homolog of CD28, and has a significant role in the maintenance of self-tolerance in the periphery, as shown by initial studies using CTLA-4 knockout mice. These mice have an extreme autoimmune-like phenotype, and are moribund within a month of being born. This fatal phenotype is marked by immune infiltration as well as inflammation of a variety of organs, including the pancreas, heart, liver, and lungs[6,7]. As a homolog of CD28, it is perhaps unsurprising that CTLA-4 binds to CD80 and CD86, the main ligands of CD28. However, while CD28

binding to CD80 or CD86 provides a stimulatory signal to the T cell, CTLA-4 binding these ligands provides an inhibitory signal. Given that the affinity of CTLA-4 for these ligands is significantly higher than that of CD28, CTLA-4 binding is able to outcompete its positive stimulatory counterpart[8]. In terms of signaling, CTLA-4 recruits two phosphatases, SHP2[9] and PP2A[10], to inhibit T cell receptor signaling. SHP2, when bound to CTLA4 within the immune synapse, is responsible for dephosphorylating the CD3ζ chain, decreasing the TCR signaling potential by blocking the activity of FYN and LCK, and decreasing the recruitment of ZAP70[9]. Downstream of the TCR signaling complex, PP2A decreases the phosphorylation of AKT[11], further dampening the signals being generated by TCR engagement, and preventing $CD8^+$ T cell activation.

Programmed Death-1

Programmed Death-1, or PD-1, is a 55 kD transmembrane protein, which, like CTLA-4, is involved in the prevention of $CD8^+$ T cell activation[12,13]. However, unlike CTLA-4, PD-1 knockout mice have a less extreme phenotype, where older animals may develop mild lupus-like autoimmunity[14] or dilated cardiomyopathy[15], depending on the background of the animal. Therefore, if CTLA-4 is an on/off switch, PD-1 is more like a rheostat for fine tuning the immune response. PD-1 binds to two, distinct ligands with unique expression patterns, named PD-L1 and PD-L2 (also known as B7-H1 and B7-DC). PD-L1 expression can be induced on most cell types, including both immune derived cells as well as epithelial and endothelial cells of peripheral organs. Expression of PD-L1 can be increased by stimulation with IFNγ or with type I interferons. In this way, PD-L1 plays a role in general protection of host cells from $CD8^+$ T cell targeting. In contrast, PD-L2 expression was originally thought to be strictly on professional antigen

presenting cells. However, some recent data have suggested that PD-L2 may be expressed on certain prostate and neurological cell lines, and can be up regulated in response to IFNγ (Nirschl, Martin, unpublished results). Expression of PD-1 on $CD8^+$ T cells is tightly regulated, and no PD-1 is found on naïve $CD8^+$ T cells due to high promoter methylation[16]. PD-1 protein is subsequently up regulated during T cell activation; however, in this work we show that PD-1 expression may not be a necessary consequence of $CD8^+$ T cell activation, but rather is controlled by the programming of the presenting dendritic cell. Further research will be necessary to determine the exact nature of the proteins responsible for controlling PD-1 expression on T cells. Nevertheless, continuous PD-1 expression has also been found on $CD4^+$ and $CD8^+$ T cells facing chronic infections, such as LCMV, or during chronic antigen exposure, such as during the progression of cancer. These features make PD-1 a promising therapeutic target, as will be discussed below.

PD-1 signaling is similar to CTLA-4[17] signaling, though evidence of combinatorial effects suggests that these proteins do not have completely overlapping functions[18,19]. PD-1 contains two well documented cytoplasmic motifs, an Immuno-Tyrosine Inhibitory Motif (ITIM) and an Immuno-Tyrosine Switch Motif (ITSM). Interestingly, PD-1 utilizes only the ITSM motif to recruit the phosphatases SHP-1 and SHP-2, although only SHP-2 recruitment has been confirmed *in vivo*[20]. The recruitment of SHP-2 to the immunological synapse by PD-1 results in dephosphorylation of the CD3ζ[21] chain, and subsequently decreases TCR signaling.

Lymphocyte Activation Gene-3

A third immune checkpoint protein that regulates $CD8^+$ T cells is Lymphocyte Activation Gene-3, or LAG-3[22]. LAG-3 is a CD4 homolog[23], and as such, has also been found to bind to MHC II[24]. Due to the restrictive nature of MHC II expression, LAG-3 has a milder regulatory role than either CTLA-4 or PD-1. This has been confirmed in LAG-3 knockout animals, where no overt, autoimmune-like phenotype has been reported[25]. However, in models where autoimmunity is initiated, such as the Non-Obese Diabetic (NOD) model, LAG-3 knockout animals have more pronounced $CD4^+$ and $CD8^+$ T cell infiltration of the pancreas and significantly accelerated disease, demonstrating that LAG-3 does have a role in preventing an immune response to inappropriate stimuli[26]. Further research has also shown that LAG-3 knockout animals have increased expansion of T cells in response to SEB activation, *in vivo* peptide stimulation, and to *Sendai Virus*, suggesting that LAG-3 may also play role in the regulation of a developing, appropriate immune response[27]. Current work in our lab has also suggested that LAG-3 expression allows for activated $CD4^+$ T cells to be suppressed by $CD4^+$ regulatory T cells (Tregs) (Durham, Nirschl Unpublished Data), which fits in the above models as Tregs are important in the maintenance of peripheral tolerance, as well as is modulating the scope of the adaptive immune response. Currently, it is known that LAG-3 has a cytoplasmic KIEELE domain[27], and that this domain is required for LAG-3 function. Furthermore, we have recently found that STAT5 and IL-2 are required for LAG-3 to regulate an immune response during homeostatic proliferation, and that LAG-3 blockade can decrease the threshold for STAT5 phosphorylation in response to

peptide stimulation. These data suggest that LAG-3 may be signaling through STAT5 to prevent T cell activation.

T Cell Immunoglobulin Mucin-3

One final checkpoint protein that has been more recently studied in the context of cancer is T cell Immunoglobulin Mucin-3, or TIM-3. TIM-3, like the other checkpoint proteins, is an extracellular receptor protein, and contains both immunoglobulin and mucin domains. Like LAG-3, TIM-3 knockout mice do not show an overt, auto-immune like phenotype[28], but are more prone to experimental autoimmune disease induction, such as in Non-Obese Diabetic Mice (NOD)[28], or the initiation of Experimental Autoimmune Encephalitis[29] (EAE). One of the major ligands for TIM-3 is a secreted lectin named Galectin-9. Galectin-9 administration *in vitro* has been shown to cause death of T_h1 $CD4^+$ T cells in a TIM-3 dependent manner[30]. In models of EAE, the disease phenotype was significantly reduced by the administration of Galectin-9, which was found to result in the deletion of the disease causing, IFNγ expressing $CD4^+$ T cells[29], correlating with the *in vitro* data. Like LAG-3 signaling, only a few parts of the TIM-3 signaling pathway in T cells are known. Current data suggests that the protein Bat3 acts as an off switch for TIM3 signaling, and continuously protects the T cell from the inhibitory signaling pathway when no TIM-3 ligands are present[31]. However, when TIM-3 binds Galectin-9, or one of its other ligands, Y265 is phosphorylated on the cytoplasmic end of TIM-3 by inducible T cell kinase (ITK)[32], displacing Bat3 and allowing TIM-3 signaling to decrease IFNγ production, hinder proliferation, and perhaps ultimately result in cell death.

Combinatorial Checkpoint Expression

Taken alone, each of these checkpoint proteins has a clear role in the management of the immune system. Furthermore, the controlled expression of the ligands of these checkpoint proteins on dendritic cells suggests that one major point of immune regulation is during the dendritic cell:T cell priming interaction. However, the complexity of these models has increased in recent years, as it has become clear that in many cases, combinations of checkpoint proteins are being expressed, especially in the case of chronic infections and cancer. In a seminal study using LCMV, Wherry et. al. showed that exhausted $CD8^+$ T cells co-express multiple checkpoint proteins, including PD-1, LAG-3, 2B4, and CD160[33]. Co-expression of multiple checkpoint proteins correlated with decreased effector cytokine production and lytic function. Other research has also suggested that PD-1 and TIM-3 are also co-expressed during chronic LCMV infection, and that co-expression of these two checkpoints also correlates with decreased $CD8^+$ T cell function[34].

In cancer, the expression of multiple checkpoints on tumor infiltrating lymphocytes has made combinatorial checkpoint blockade an attractive target for cancer treatment. Indeed, studies publishing the benefits of single checkpoint blockade by blocking either the CTLA-4[35] or PD-l[36–38] signaling pathways in clinical settings have been documented in the past decade, leading ultimately to CTLA-4 blockade being the first FDA approved checkpoint therapy. Preclinically, the first combinatorial blockade studies were done combining PD-1 and CTLA-4[39,40]. In these studies, while individual blockade showed some anti-tumor immune responses, combinatorial blockade was far superior. In mechanistic studies, combining PD-1 and CTLA-4 blockade with an

irradiated tumor vaccine resulted in increases of both the $CD8^+$ and $CD4^+$ effector populations when compared to regulatory T cells[41]. While these studies were initially done in models of melanoma, the anti-tumor treatment effects of combined PD-1 and CTLA-4 blockade have been repeated in models of bladder cancer[18], as well as ovarian cancer[42], demonstrating that combinatorial blockade of PD-1 and CTLA-4 may have potential across a broad range of tumor types.

TIM-3 expression on tumor infiltrating lymphocytes has been confirmed in several preclinical tumor models as well, including CT26 colon carcinoma, 4T1 mammary carcinoma, and B16 melanoma[43]. Interestingly, TIM-3 was found almost universally co-expressed with PD-1, and combined PD-1 and TIM-3 blockade proved to be the most effective immunotherapy in terms of anti-tumor immunity. Furthermore, mechanistically, expression of multiple checkpoints, in this case TIM-3 and PD-1, was found to correlate with decreased expression of effector cytokines, such as IFNγ, IL-2, and TNFα, as was seen previously in the viral models of chronic infection.

Co-expression of PD-1 and LAG-3 represents an important mechanism of self-tolerance, as these two checkpoints are co-expressed during the recognition of self-antigen[44]. These findings were extended through the generation of mice that express neither PD-1 nor LAG-3 (DKO mice). In these animals, the normally subtle autoimmune phenotypes of the single knockouts is replaced with extreme immune infiltration of the heart, lungs, and liver[45,46]. Additionally, most of these animals are moribund within 6-8 weeks after birth. Important for cancer therapy, we found PD-1 and LAG-3 were co-expressed on $CD4^+$ and $CD8^+$ tumor infiltrating lymphocytes in B16 melanoma, SA1N fibrosarcoma, and MC38 colon carcinoma. Using SA1N and MC38, we found that

tumors implanted on DKO mice were mostly rejected, while tumor growth was only delayed in PD-1 single knockout mice and left relatively unaffected by the absence of LAG-3. In a more clinically relevant model, we treated established SA1N and MC38 tumors with monoclonal antibodies against PD-1, LAG-3, or both. Similar to the knockout experiments, PD-1 or LAG-3 monotherapy did delay tumor growth, but combinatorial blockade resulted in rejection of the majority of the established tumors. Furthermore, and perhaps most importantly, combinatorial blockade of PD-1 and LAG-3 as a treatment model did not induce the extreme auto-immune like infiltration seen in the knockout animals[45].

In the clinic, there is mounting evidence of combinatorial expression of checkpoint proteins. Cancer testis antigen NY-ESO-1 specific T cells co-express PD-1 and LAG-3 in ovarian cancer patients[47], while the TILs of melanoma patients often co-express PD-1 and Tim-3atients[48]. Early clinical studies utilizing CTLA-4 blockade, or Ipilimumab, were initially very exciting, as approximately 15% of melanoma patients showed objective responses[35]. Moreover, those patients that did respond showed surprisingly long lasting tumor control, which has become a hallmark of successful immunotherapy. Likewise, anti-PD-1 therapy, or Nivolumab, has shown responses in about 30% of melanoma patients, and those responses are often durable for years[36]. Recent results combining these two blockades have demonstrated similar combinatorial effects as seen in preclinical models. Strikingly, patients who responded to combinatorial therapy (about 53%) had 80% decreases in tumor size by the first scheduled assessment, showing a rapid anti-tumor response[49]. Only time will tell if these responses will

continue, but data from the CTLA-4 and PD-1 individual blockade trials suggest that many of these responses will be long lived.

Cancer Vaccines

While checkpoint blockade represents the reactivation of a previously generated response, in certain pathologies, such as breast, prostate, and kidney, the percentage of tumor specific T cells found in patients is relatively low. However, in other cancer types, such as melanoma, the frequency of tumor specific T cells is fairly sizable. Currently, it is unknown why only some cancers develop significant $CD8^+$ T cell populations, but the presence of a significant population of tumor specific T cells is important for the generation of anti-tumor immunity[50,51]. Additionally, without tumor specific T cells, the efficacy of checkpoint blockade would be significantly hindered. Luckily, there is already a well-established tool for the generation of an adaptive immune response in the clinic, known as a vaccination. However, while current protective clinical vaccines are highly effective, they do not have to subvert tumor- induced tolerance, and are given prophylactically to patients with fully intact and competent immune systems. Anti-cancer vaccines must be developed to break tumor induced tolerance, and must show clinical benefit in a therapeutic setting. Broadly, cancer vaccines can be divided into three categories; DNA Vaccinations, Cell Based Vaccinations, and Live-Attenuated Vaccinations. Each of these classes of vaccine has their own strengths and weaknesses, as well as unique mechanisms of action, as will be discussed below.

DNA Vaccines

As their name suggests, DNA Vaccines are based on the introduction of naked genetic material into the host, with the goal of having this gene or genes expressed to generate a potent immune response[52–54]. One of the primary practical advantages of DNA based vaccinations is their potential for easy, large scale generation. Furthermore, DNA is extremely stable, and thus could be easily stored and transported with little need for technical support. One of major clinical and scientific advantages of DNA based vaccinations is the unlimited possibilities for encoding fusion proteins that both include targets and their subsequent adjuvants, allowing for the generation of a protein that did not previously exist but is both targetable and immune-stimulatory. Yet, despite these advantages, DNA vaccines have not been particularly potent in clinical trials[55], likely due to the small amount of target antigen they produce, as well as the likelihood that naked DNA will be rejected instead of transcribed by the host machinery.

Mechanisms of Action

In terms of design, DNA vaccines often utilize a bacterial plasmid backbone, including hypomethylated CpG motifs. These motifs, along with other pathogen associated features of the bacterial backbone are able to induce strong innate immune responses at the site of vaccination, such as increased production of IL-6, IL-12, TNFα, IFNγ, IFNβ, and IFNα[56,57]. Originally, the innate immune response to DNA vaccines was thought to be driven by TLR9 signaling, which binds to hypomethylated CpG motifs, such as those in a bacterial or viral genome. However, recent studies have shown that DNA vaccines are equally potent in TLR9 knockout animals, and that the innate immune

response may instead be driven by cytoplasmic innate immune sensors such as STING or TBK-1[58]. These features also help to polarize the resulting $CD4^+$ T cell response to a T_h1 response, helping to ultimately generate effective $CD8^+$ T cell responses.

The molecular mechanism of DNA uptake and transcription are poorly understood, though methods of transfection, such as electroporation or utilization of a gene gun, have been found to increase efficacy of these vaccines[59]. Furthermore, which cell population transcribes and presents the proteins encoded by DNA vaccines is still unknown, though there are two main hypotheses that are currently being investigated. The first is that DNA vaccines directly transfect dendritic cells or other professional antigen presenting populations. Some evidence of this has been generated by the inclusion of survival factor plasmids in the initial transfection, theoretically prolonging dendritic cell life and capability for antigen presentation. In these studies, inclusion of a second plasmid containing the anti-apoptotic proteins BCL-$_X$l resulted in increased numbers of plasmid containing dendritic cells surviving in the draining lymph nodes following vaccination[60]. Furthermore, co-culture of dendritic cells from the draining lymph nodes of mice receiving both plasmids with naïve $CD8^+$ T cells primed those T cells to produce significantly more IFNγ, suggesting that these dendritic cells were more potent inducers of $CD8^+$ T cell immunity. One caveat of this study was the utilization of a gene gun, which is known to increase dendritic cell transfection when compared to naked DNA alone.

The second possible hypothesis is that non antigen presenting cells at the site of vaccination form a type of antigen depot, which then undergoes apoptosis, and the debris is cross presented by professional antigen presenting cells, likely those of the $CD8\alpha^+$

dendritic cell lineage. In support of this hypothesis, it has also been demonstrated that the inclusion of a Fas containing plasmid with a DNA vaccine plasmid increases both the concentration of the target protein in the draining lymph node as well as the resulting cytolytic function of whole splenocytes when anti-FAS antibody is included in the treatment scheme[61]. Other studies have shown that co-transfection of target antigen plasmids with a plasmid containing fully functional caspase 3 generated larger numbers of target specific $CD8^+$ T cells as determined by ELISPOT[62]. Along with the Fas based studies, these data suggest that the plasmid containing cells needed to undergo cell death to provide functional immunity. Given the relatively nonspecific nature of these vaccinations, it is likely that both mechanisms play some role in the generation of an immune response though future research will be important in determining the exact functional mechanism of action.

Fusion Proteins and Their Importance

One of the primary disadvantages to DNA vaccinations is their relative inefficiency due to a large number of molecular steps between vaccination and the subsequent immune response to a specific protein.. Indeed, antigens must first be translated to a protein form, and then processed by professional antigen presenting cells to have any hope of generating an immune response. Morever, transfection efficiency is relatively low *in vivo*, due to the natural, anti-viral response that mammalian host cells have to foreign nucleic acids. The end result is low overall amounts of target protein being transcribed. To help enrich the target protein into professional antigen presenting cells, the tumor antigens in DNA vaccines may be fused to a protein that will help it home to professional antigen presenting cells[63]. For example, fusion of the hepatitis B

virus e (HBVe) antigen to a human IgG constant region results in significantly increased levels presentation of the HBVe protein in host dendritic cells, despite similar levels of transfection efficiency. Dendritic cells from mice receiving the fusion protein plasmid were more potent inducers of T cells responses when adoptively transferred into new hosts, suggesting that they may also be more mature[64]. Interestingly, other fusions of immune proteins have been shown to generate anti-tumor immunity in the setting of B cell lymphoma. In these models, the tumor and self-antigen idiotype of the B cell receptor (ID) is normally weakly immunogenic. However, when a DNA vaccine fusing CTLA-4 to ID was administered prophylactically, ID specific T cell responses could be generated, and mice were protected from lethal tumor challenge. Furthermore, mutants of this fusion construct that could no longer bind CD80 or CD86 were no longer able to generate robust T cell responses to ID, suggesting that the generated fusion protein works by binding to antigen presenting cells and being internalized through receptor mediated endocytosis[65]. Many other fusion proteins have been found to localize target proteins into antigen presenting cells, including heat shock proteins[66], molecules of the complement cascade pathway (C3d)[67], or the β-defensin proteins[68].

Stimulatory Molecules and DNA Vaccines

In addition to the use of fusion proteins, it is possible to include chemokines, or trafficking molecules to attract professional antigen presenting cells to the site of vaccination. As proof of principle, the addition of a plasmid encoding the chemokine MIP-1β to a DNA vaccine plasmid containing HIV gag protein resulted in significantly increased cellular infiltration at the site of vaccination, as well as the generation of protective immunity against GAG expressing Vaccinia Virus[69]. Additionally, inclusion of

a plasmid encoding CCL19 with the DNA vaccine plasmid encoding β-galactosidose has been found to stunt growth of the fibrosarcoma line MCA205 expressing β-galactosidose as a target antigen, as well as skew responding $CD4^+$ T cells towards a T_h1 phenotype[70].

Clinical Results

Pre-clinical results with DNA vaccines were initially promising, though upon current review it is clear that these vaccines were predominantly tried in settings of lymphomas and other humoral immunity, which are historically easier to treat than solid tumors. Furthermore, many of these DNA vaccines were used prophylactically to prevent the implantation of cancer, which is not how they were going to be tested clinically. To date, several clinical trials have been done utilizing DNA vaccination platforms[55], and all have confirmed safety of this approach, as well as the generation of an immunological response in the form of tumor specific $CD4^+$ and $CD8^+$ T cells, as well as formation of antibodies in response to the vaccination[71–74]. However, these clinical trials have had little effectiveness in the generation of potent anti-tumor immunity, possibly due to the difficulties of integrating foreign DNA into host cells, or of concentrating the target protein in the appropriate cell types. It is also plausible that the immunocompromised status of the patients receiving these trials makes it difficult to generate potent anti-tumor immunity, though other vaccine vectors have had more success with a similar patient population.

Cell Based Vaccines

Unlike DNA vaccines, which need to integrate into the host machinery for effectiveness, cell based vaccination strategies include all the machinery necessary for

protein translation and folding. Typically, cell based vaccine strategies follow a general regimen involving the *in vitro* manipulation of a cell population prior to introducing the newly modified cells to the patient as a vaccine. The type of cell used varies from genetically modified tumor cells, either from the patient or from a cell line, to protein pulsed and matured dendritic cells. In both settings, antigen dose is significantly higher than what is seen with DNA vaccines, and the generation of an immune response is not impaired by natural host aversion to the vaccine platform, as has been seen when trying to transfect cells *in vivo* with naked DNA. However, this vaccine type has its own set of disadvantages. First off, and perhaps most importantly, cell based vaccination strategies are poorly immunogenic when compared to live-attenuated vaccines. Cell based vaccination strategies are missing adjuvant and pathogen associated molecular patterns, as they are not derived from pathogens. As such, it may be difficult for them to break tumor induced tolerance to certain antigens. Furthermore, since they are live cells, production, maintenance, and storage all require increased levels of technical expertise and cost. Finally, some cell based vaccines utilize the patient's own cells, either tumor cells modified to become immunogenic, or immune cells modified to stimulate a potent $CD8^+$ T cell response. These strategies provide individualized treatment to patients, but take exponentially more time, technical expertise, and funding to develop and use. Yet, despite these disadvantages, two cell based vaccine strategies have seen promising clinical results; namely GVAX and Sipuleucel-T.

Cancer Cell Based Vaccines: GVAX

Early in cancer immunotherapy, few tumor associated antigens had been identified, and even today, the number of tumor associated antigens identified likely pales

in comparison to the number of mutations being expressed by an individual tumor. Therefore, to circumvent the need to identify an individual target, whole cell vaccines were investigated. However, as stated previously, due to immune editing, many cell lines were poorly immunogenic, like clinical disease. Yet, it stood to reason that it was possible to modify these tumor lines *in vitro* to restore their immunogenicity, perhaps through the production of stimulatory cytokines. Therefore, Dranoff et. al performed a seminal study that introduced overexpression of different stimulatory cytokines into the melanoma cell line B16-F10, which was known to be poorly immunogenic[75]. After a heavy dose of radiation, the now cytokine expressing tumor lines were reintroduced to the host animals as a vaccine against the parental tumor cell line. Of the cytokines examined, GM-CSF generated the most potent anti-tumor immunity. These groundbreaking results were directly responsible for the generation of many different tumor lines, all expressing GM-CSF[76–79]. Today, the process of overexpressing GM-CSF in a tumor line, subsequent irradiation, and reintroduction into a host as a vaccine is known as GVAX[80,81].

Mechanism of Action

GM-CSF is a cytokine that has been shown to induce the generation of potent antigen presenting cells[82], though more recent studies suggest that GM-CSF treatment may selectively expand certain subsets of dendritic cells more than others[83]. GM-CSF produced by tumor vaccinations results in increased dendritic cell trafficking to the site of vaccination, as well as increased expression of maturation markers on dendritic cells[84]. Therefore, when the GM-CSF producing tumor cells that have been reintroduced undergo apoptosis due to extensive radiation damage, these cells will release danger signals,

which, along with tumor antigens, will be picked up by the newly recruited dendritic cells. Indeed, vaccination with GM-CSF secreting tumor cells has been found to increase dendritic cell infiltration of tumors, as well as provide protective immunity against rechallenge with parental B16-F10[84]. Interestingly, GM-CSF secreting tumors seemed to expand out the CD8α^- dendritic cell subset in the spleen, and these cells expressed increased levels of CD80, and CD1d, suggesting increased maturation. Finally, the potency of the GVAX approach is not limited to melanoma cell lines, but has been preclinically confirmed in many different types of cancer, including lymphomas, leukemias, mammary carcinomas, and colon carcinomas to name few[76–79,85].

Clinical Results

Clinically, several phase I and phase II trials have been completed and reported utilizing autologous vaccination strategies[80]. In these trials, including patients with melanoma, prostate cancer, and non-small cell lung cancer, the majority of patients had delayed type hypersensitivity reactions, as well as cellular infiltration of the vaccination site by dendritic cells, macrophages and eosinophils[86–88]. Furthermore, in trials designed to study efficacy of the vaccines, approximately 20% of patients showed either stable disease or mixed responses, suggesting that this approach could have significant therapeutic potential.

In the early GVAX trials, development of the vaccine platform, namely the creation of a GM-CSF secreting autologous tumor line from the patient's own resected tumor, took between eight to ten weeks. During this time, many patients had their disease progress, and become subsequently ineligible for vaccination. Therefore, the

development of an allogeneic, off-the-shelf treatment utilizing the GVAX platform was developed. While the specificity to the patient was lost, many tumor antigens are shared within a tumor type, and some universal antigens have been found across tumor types, thus still maintaining the potential for a multi-antigen response. Therefore, allogeneic GM-CSF secreting lines were developed and tested clinically. One of the early studies in pancreatic cancer found that vaccination following surgical resection increased survival by about 5 months when compared to historical controls. Another study in pancreatic cancer did not find a survival benefit, but did find that 38 out of 43 patients analyzed generated $CD8^+$ T cell responses to at least one new mesothelin peptide[89]. Yet another study, using a $Her2^+$ breast cancer GVAX vaccine, found that combinations of GVAX with cyclophosphamide treatment could generate HER2 specific antibody responses in 32% of patient treated[90]. Together, these data showed promising results through the use of genetically modified whole cell vaccines, and confirm that the immune system is capable of responding to tumor antigens, when shown them in the proper context.

Dendritic Cell Based Vaccines: Sipuleucel-T

Another type of cell based vaccination involves the modification of not the tumor cells, but of the patients own dendritic cells[91]. In this vaccine, lymphocytes are removed from the patient's blood, before being cultured *in vitro* in the presence of target antigen as well as a maturation stimulus. This matures and expands the population of dendritic cells, and loads them with a specific target before they are infused back into patients. This method provides the functional antigen presenting population necessary for the generation of an adaptive immune response. There are several advantages to the use of dendritic cells for a cell based vaccine. One of the major advantages is that *in vitro*

manipulation of the dendritic cells directly bypasses any potential for inadequate dendritic cell activation, be it due to suboptimal dosing, or lack of pattern associated molecular patterns *in vivo*. Furthermore, it also allows for easy manipulation of the target antigen, simply by adding a different antigen to the *in vitro* mix. However, this vaccine strategy is only capable of mounting a single-antigen immune response, specifically directed at the target protein. This contrasts with GVAX based approaches, where the whole proteome of the tumor is presented in a vaccination context. Also, as was seen in the autologous GVAX approaches, the use of a single patients own dendritic cells requires high levels of technical expertise, as well as special care in handling, culturing, and storing. This increases both the time and cost associated with this type of vaccine. Finally, this approach requires the patients to have a healthy enough dendritic cell population to successfully culture a response *in vitro*, which may prove difficult in some older, more immunocompromised patients.

Mechanism of Action

Early work in preclinical models found that isolation of dendritic cells, *ex vivo* antigen pulsing and maturation, and subsequent reinjection of those dendritic cells into their original hosts resulted in the generation of potent anti-tumor immunity in a variety of models, including B cell lymphoma, fibrosarcoma, breast carcinoma, and lung carcinomas[92–94]. To generate protective immunity using this strategy, dendritic cells are crucial as pulsing other cell types, such as B cells, has no vaccination benefit[92]. Furthermore, transfer of peptide loaded dendritic cells can generate tumor specific antibodies[92], as well as $CD4^+$ and $CD8^+$ T cell responses[93,94], resulting in tumor lysis. Finally, dendritic cell vaccines have also been found to produce anti-tumor immunity in

established tumors, when treated *in vitro* with GM-CSF and acid eluted, unfractionated peptides from resected tumor[95]. In this model, it would actually be possible to mount a multi-epitope immune response. When utilizing dendritic cell vaccines, $CD4^+$ and $CD8^+$ T cells are required for the generation of anti-tumor immunity, and transfer of sera does not provide protection. Furthermore, blockade of CD28 signaling by the administration of a CTLA4-IG fusion protein with the dendritic cell vaccine prevents therapeutic effect[95]. Together, these data suggest a model by which dendritic cells are functionally matured in the presence of target antigens *in vitro*. During this maturation process, co-stimulatory signals, such as CD80 and CD86 are generated, providing both signal one, in the form of target antigen, and signal two, in the form of co-stimulatory molecules. Together, these signals result in the generation of a potent T cell response, which has been found to be critical for the rejection of solid tumors. The culmination of these preclinical data was the development of the clinical treatment, Sipuleucel-T.

Clinical Results

Clinically, Sipuleucel-T, also known as Provenge, was the first cancer vaccine approved by the FDA, making it a landmark treatment for minimally symptomatic metastatic castration-resistant prostate cancer (CRPC). Broadly, this vaccine involves the removal of the patient's dendritic cells from peripheral blood, followed by *in vitro* treatment with a fusion protein combining GM-CSF with the tumor associated antigen, Prostatic Acid Phosphatase (PAP). Following *in vitro* treatment, these dendritic cells are transferred back into their original host. In three clinical trials, Sipuleucel T was found to extend the lives of patients by about 4 months[96,97,98], though statistical significance was not reached in the first trial. Furthermore, as was seen in the preclinical data, PAP

specific T cell responses were generated by this vaccine in about 28% of patients treated, further validating the scientific rationale behind these treatments.

Live, Attenuated Vaccine Vectors

The last category of cancer vaccine is known as a live-attenuated vaccination. As the name would suggest, these vaccines are metabolically active, though weakened, pathogenic vectors. Many vaccinations currently utilized in the clinic fall into this category, including the vaccinations for mumps, measles, and chickenpox. To generate viral live-attenuated vaccines, wildtype viruses are repeatedly grown in suboptimal culture conditions, including cells they replicate poorly in, selecting for viral products that grow better in these suboptimal conditions and subsequently weakening their virulence in the original host. Bacterial live-attenuated strains are produced through the directed deletion of certain genes in the bacterial genome[99]. Perhaps the most important advantage of live-attenuated vaccines when compared to the previous two types is that live-attenuated vaccinations are the most foreign vector. Thus, these vaccines are capable of generating potent cellular and humoral immunity, and clinically, are typically potent as a single dose vaccination for life. Like the DNA vaccines, live-attenuated vaccines can also be easily genetically modified to include target antigens or stimulatory proteins. Additionally, live-attenuated vaccines can be made into off-the-shelf therapies, and are translatable into large scale production. Yet, since the viral or bacterial vector is foreign, live-attenuated vaccines can only present a limited number of pre-selected targets. Furthermore, introduction of some targets in bacterial vaccine vectors can affect their potency as the lifecycle of the bacteria is interrupted. Live-attenuated vaccines also have a unique disadvantage, in that they are still virulent, and have the potential to replicate

and infect their host. This has often been addressed by the use of inactivated vaccinations, where the vector is killed either through chemical treatment, heat, or radiation. While killed vaccines have no pathogenicity once killed, they also stimulate a weaker immune response, and generally require booster treatment to generate fully protective immunity. Finally, one unique property of live-attenuated vaccines, that can be either an advantage or disadvantage, is that the mechanism of action will be unique to each individual vector. Therefore, the potency of one target antigen in a certain live-attenuated vector, such as Vaccinia Virus, may be completely different from the potency of that same target in a different vector, such as *Listeria Monocytogenes*.

Vaccinia Virus Vectors

Vaccinia Virus, a member of the poxvirus family[100], is a double stranded DNA virus that has been one of the major success stories in the field of Immunology, and its widespread use is largely responsible for the eradication of smallpox. As with many other live-attenuated vaccines, Vaccinia Virus was generated by passaging the parental virus through a series of hostile culture conditions, resulting in a strain of virus that is metabolically active, but grows poorly in humans (and mice). I*n vivo*, our group and others have shown that Vaccinia Virus given intravenously is likely presented to the adaptive immune system only by $CD8\alpha^+$ conventional dendritic cells[101]. However, despite its attenuated status, the adaptive immune response, specifically $CD4^+$ T cells, is crucial to the clearance of Vaccinia[102].

Mechanistically, Vaccinia Virus has also been found to produce a series of proteins that interfere with the immune response being generated. Once in the cell,

Vaccinia produces viral protein A46, stopping the infected cell from recognizing its infection by binding signaling adaptor proteins of the TLR and RLR signaling pathway through its TIR domain[103,104]. A46 can bind MYD88, MAL, TRIF[105], and TRAM, preventing TLR signaling, among other innate pathways, from responding to the infection. Viral protein A52 binds to IRAK and TRAF6 signaling proteins interfering with downstream NFkB activation[103,106], while protein K7 inhibits viral recognition pathways from producing IFNβ[107]. These proteins, along with a series of other viral products[108] modify the immune system's capability to respond to Vaccinia. These interactions with the immune system are likely what makes different live-attenuated vaccination vectors different in terms of the generation of adaptive immunity.

Due to its foreign and infectious nature, Vaccinia is capable of generating a strong immune response. It is also fairly easy to genetically manipulate, making it a good vector for the addition of tumor associated antigens. A variety of clinical tumor associated antigens have been added to a Vaccinia Virus vector, including Carcinoembryonic Antigen (CEA)[109–111], Prostate Specific Antigen (PSA)[112–114], melan-A/MART-1, Tyrosinase, and gp100[115,116]. In each of these trials, Vaccinia based vectors were able to induce tumor associated antigen specific $CD8^+$ T cell responses as well as antibodies against the targeted protein. However, the clinical results were often limited to stable disease in the best case. Of note, when Vaccinia Virus was used as a vector to immunize against the E2 protein of HPV, remarkably 34 out of 36 patients treated showed elimination of precancerous lesions, along with the generation of HPV E2 specific antibodies, and a significant reduction in HPV viral load[117]. These data suggest that Vaccinia may be a good live-attenuated vector for vaccination against other viruses, such

as HPV. In the case of HPV^+ cancers, viral proteins are capable of driving uncontrolled cell division, and thus, clearance of the virus is often directly correlated with regression in these settings. However, the question remains why Vaccinia vector based vaccination seem to produce $CD8^+$ T cell responses that are incapable of tumor rejection in clinical diseases that are not driven by chronic infection.

Listeria Monocytogenes Vectors

Listeria Monocytogenes is a gram positive, rod shaped, intracellular bacteria. Similar to Vaccinia, *in vitro*, *Listeria* is capable of infecting a wide variety of cell types. Nevertheless, early research utilizing *Listeria Monocytogenes* as a model pathogen demonstrated that the deletion of the $CD11c^+$ conventional dendritic cell population significantly decreased the resulting $CD8^+$ T cell response[118]. Further research has shown the $CD8\alpha^+$ dendritic cells are required for *Listeria Monocytogenes*, given intravenously, to enter the spleen[119,120]. However, the exact cell type responsible for priming the adaptive immune response has not been fully elucidated. In terms of the molecular response, *Listeria Monocytogenes* is known to be recognized by the TLR2[121], TLR4, RIG-I[122,123], STING[124], and NOD-like[125] pathways. Each of these pathways has been shown to be critical to the clearance of wild type *Listeria*, though none are required for the production of IFNβ that is a hallmark of *Listeria* infection[126]. However, production of IFNβ does require interferon response factor 3 (IFR3) and Tank Binding Kinase-1 (TBK-1)[127–129], though it is possible that all the above mechanisms result in IRF3 and TBK-1 signaling, and thus overlap to sense intracellular *Listeria*. Furthermore, myeloid dendritic cells only produce IFNβ when the *Listeria* being used is metabolically active[130],

suggesting that one of the key mechanisms to the generation of an immune response to *Listeria* is the pathogen's ability to get into the host cell's cytoplasm. Further supporting this hypothesis is the fact that mice with the type I interferon receptor knocked out are resistant to *Listeria* infection[131].

To avoid the immune system, *Listeria* has also developed a series of proteins that allow it evade the normal immune response during its lifecycle. Following uptake into an endocytic vesicle, the bacterium expresses Listeriolysin O, which is a pore forming protein that allows the *Listeria* to avoid degradation in the vacuole. LLO is maximally active at a pH of 5.5[132], allowing for the bulk of its activity to occur within the acidic endosomes of host cells, without punching holes in the cell's plasma membrane. After lysing the endosome, the bacterium is released into the cytoplasm of the infected cell, where it would normally be targeted by the autophagocitic machinery of the host cell. To prevent this, *Listeria* has a protein named Actin Assembly-inducing Protein A (ActA), which acts as a molecular mimic of the Wiskott-Aldrich Syndrome Protein, recruiting the host Actin-Related Protein 2 and 3 complex (Arp2/3) to form actin tails[133]. The force generated by the assembly of actin tails push the bacterium throughout the cell, preventing autophagy[134]. Furthermore, actin tails allow for the bacterium to travel from the initially infected cell into neighboring cells, allowing for the spread of infection without exposing the bacteria to the extracellular environment. Finally, the process of bacterial spreading is enhanced when the neighboring cells are producing nitric oxide, suggesting that *Listeria* may have adapted to thrive in the midst of an immune response[135]. Unlike Vaccinia, which seeks to hide from the immune system through the

inhibition of activation pathways, *Listeria* seems to benefit from the generation of an immune response and instead has found molecular mechanisms to escape rejection.

In the wildtype form, *Listeria* infections can be cleared by healthy hosts in a manner that depends on the formation of an adaptive immune response. However, as previously discussed, *Listeria* also has a variety of mechanisms to avoid the immune system, and so must be modified prior to its use as a vaccine. Fortunately, like Vaccinia, *Listeria* has been found to be amenable to genetic modification. By knocking out the bacterial proteins ActA and Internalin B (IntB), the virulence of a *Listeria* vector is decreased 1000 fold when compared to the wild type bacteria[136]. With these two genes deleted, the *Listeria* vaccine vector is more likely to enter phagocytic cells, such as a dendritic cells or macrophages, and the bacterium can no longer spread between cells, limiting its infectious potential. Preclinically, a number of target tumor associated antigens have been added to this live-attenuated *Listeria* vector, including HPV16 E7[137], PSA[138], and the melanoma antigen TRP-2[139]. In these models, *Listeria* based vectors have been found to generate $CD8^+$ T cells capable of producing IFNγ. with limited antibody formation. This is potentially relevant, since it limits the likelihood of neutralizing antibody formation and provides scientific rationale for repeat dosing to generate the most potent anti-tumor immunity. Additionally, anti-tumor immunity had been found in both prophylactic and therapeutic settings, and protective memory responses were generated. Finally, previous work from our group has shown the *Listeria* based vaccines provide enough stimulation to rescue $CD8^+$ T cell responses to self-proteins, including increased divisions and cytolytic function, as well as the generation of increased effector

cytokines (Goldberg, unpublished data). These data demonstrate that live-attenuated *Listeria* vectors are capable of breaking tolerance to self, and potentially to tumors.

Clinically, a trial testing the safety of the live-attenuated Listeria vector has just completed[140]. This trial represents the first trial in which the *Listeria* vector had both the ActA and IntB genes knocked, though previous trials have been done using a *Listeria* vector with LLO knocked out[141]. From this trial, all tested doses of this strain of *Listeria* were well tolerated, and 37% of the initial testing cohort survived >15 months, and long term survival correlated with development of an immune response to the vaccine[140]. In both these studies, *Listeria* vaccination induced both T cell responses capable of producing IFNγ in response to both *Listeria* derived antigens as well as the target antigen being examined. While this study was not powered to test survival, these are promising results for initial studies.

Summary

The adaptive immune system is extremely powerful, and able to discriminate between targets at the molecular and cellular level. Moreover, the immunity that is generated is often sterilizing, and provides lifelong protection in the form of memory. Research has shown that the immune system can recognize and reject cancer, and may do so more often than previously thought. However, by the time of a clinical diagnosis, the immune system has been rendered tolerant to the tumor, either through the absence of cells capable of rejecting the tumor, or their subsequent tolerization. Cancer vaccinations represent a clinical strategy by which new immune responses can be generated against the tumor, or existing ones can be boosted. On the other hand, monoclonal antibody-

mediated blockade of checkpoint proteins can be used to reverse tolerance and maintain anti-cancer responses once they have been initiated. Realistically, optimal immunotherapy treatments for cancer will likely be a combination of these two strategies, where checkpoint blockade is utilized to amplify and protect a developing immune response from cancer derived tolerance.

However, questions still remain about why certain cancer vaccines are more effective than others. What are the effects of different vaccines on dendritic cells, and how do these differences translate into different $CD8^+$ T cell responses? Do differences in dendritic cell responses correlate with anti-tumor immunity, and can these differences be utilized to design the optimal anti-cancer vaccine? How do different vaccine vectors affect checkpoint protein expression, and how can this knowledge be manipulated to ensure maximal anti-tumor immunity? Future research will be necessary to identify the molecular determinants in the professional antigen presenting cells that control the potency and characteristics of the adaptive immune response. In this work, we identify the differences in responding antigen presenting cells responsible for generating an adaptive immune response to both *Listeria* and Vaccinia. Furthermore, we identify the fact that these differences have significant effects on the resulting $CD8^+$ T cell population, and how those molecular differences may generate different functional outcomes in terms of anti-tumor immunity. Finally, we identify a previously unknown function of live-attenuated *Listeria* vaccines, whereby the vaccine allows for $CD8^+$ T cell activation without PD-1 expression. Together, these data show the important potential for overlap between checkpoint blockade and vaccines. Indeed, the effects of any vaccine vector on checkpoint protein expression need to be evaluated carefully to determine if

checkpoint protein status correlates with anti-tumor function, both preclinically as well as clinically.

CHAPTER II

GENERAL MATERIALS AND METHODS

Mice

Mice were kept and used in accordance with guidelines of the Johns Hopkins University Institutional Animal Care and Use Committee. All mice used were female mice between 8-16 weeks old. Female B6-Ly5.2 (also known as CD45.1) congenic mice were obtained from the National Cancer Institute at Fredrick. $Rag2^{-/-}$ mice on the C57/BL/6 background were purchased from Jackson Laboratory (Bar Harbor, Maine). OVA specific, OT-1 mice on a C57BL/6 ($H\text{-}2^{b}$) background were originally obtained from Dr. M. Bevan (University of Washington, Seattle, WA), and were bred at JHU onto a $Rag2^{-/-}$ background. HA specific, Clone 4 $Thy1.1^{+}$ mice were originally obtained from Dr. Linda Sherman (Scripps Research Institute, La Jolla, CA). $PD\text{-}1^{-/-}$ mice on a C57/B6 background were provided by Dr. Lienping Chen (Yale University, Connecticut, MA) with permission from Dr. T. Honjo (Kyoto University, Kyoto, Japan). B10.D2 $Thy1.2^{+}$ mice were originally purchased from Jackson Laboratory (Bar Harbor, ME) and were subsequently bred and maintained at JHU. C3-HA^{lo} mice were generated originally in our lab[142], and express Hemagglutinin driven by the rat C3 promoter, which has been found to be one of the subunits of the oligiomeric prostatic steroid binding protein. The resulting mouse expresses HA at detectable mRNA levels in the lungs, muscle, heart, pancreas, salivary gland, as well as the prostate and testes. These mice express about 15x less HA than the C3-HA^{hi} mouse. Despite decreased expression of HA, C3-HA^{lo} mice provide a tolerizing environment for HA specific CD8+ T cells, and were therefore used as a control for a non-inflammatory condition in these experiments. CAG-OVA mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Briefly, these mice express Ovalbumin under the control of the β-actin promoter, resulting in ubiquitous

expression of Ovalbumin throughout the mouse, and providing for a non-inflammatory condition for OVA specific $CD8^+$ T cells. $MyD88^{-/-}$ mice were originally created through the addition of a LoxP site to either side of exon 3 of the MyD88 gene, followed by subsequent breeding to a mouse expressing Cre protein in germ line cells. These mice were a generous gift from Dr. Franck Housseau (Johns Hopkins University, Baltimore, MD). STING KO mice, otherwise known as Golden Ticket mice, were generously provided by Dr. Young Kim (Johns Hopkins University, Baltimore, MD), who originally received these mice from Dr. Russell Vance(UC Berkley, Berkley, CA)[143]. Briefly, these mice were generated through mutagenesis with N-ethyl-N-nitrosourea, during which a single nucleotide variant T596A in the STING gene occurred, resulting in no detectable production of the STING protein[143].

Buffers

Unless otherwise stated, all cell culture was performed in RPMI 1640 (Corning, Manassas, VA) supplemented with 10% fetal bovine serum (Gemini Bio Products, Sacramento, CA), 1 moll MEM sodium pyruvate (Sigma, St. Louis, Missouri) 0.1 mM MEM nonessential amino acids, 1000 IU penicillin, 1000 IU streptomycin (Lifescience Technologies, Grand Island, New York), 4.5×10^{-5} M 2-ME (Lifescience Technologies, Grand Island, New York). Henceforth, this combination is referred to as "complete RPMI." Miltenyi isolations and extracellular staining were performed in MACs buffer. To make MACs buffer, 2mM EDTA (Lifescience Technologies, Grand Island, New York) and 0.5% Bovine Serum Albumin (Roche, Mannheim, Germany) were added to PBS (Lifescience Technologies, Grand Island, New York). For intracellular staining, cells were fixed using Fix/Perm (Ebioscience, San Diego, CA) kit and stained in Perm

Buffer (Ebioscience, San Diego, CA), which were both generated from concentrate according the manufacture's protocol. In the lung harvest experiments, lungs were fixed and bleached using Fekete's Solution, which is made by combining the following: 580mLs 95% ethanol, 200mL H_2O, 80mLs of 37% formaldehyde solution, and 40mLs of glacial acetic acid.

Cell Lines and Vaccines

Both B16-OVA and EL4-OVA were a generous gift from Dr. Hyam Levitsky. Breifly, to generate these cell lines, the parental strains EL4[144] and B16[145] were electroporated or lipofected, respectively, with a plasmid construct containing the whole chicken ovalbumin gene under the control of the human β-actin promoter, as well as resistance genes to G418 and neomycin for selection. To preserve OVA expression both B16-OVA and EL4-OVA were both grown in complete RPMI media supplemented with 0.5mg/mLG418 prior to implantation. *Listeria* expressing OVA protein was a generous gift from Dr. Pete Lauer at Aduro Biotech. Briefly, live attenuated strains of *Listeria* were generated from the 10403s strain by in-frame deletions of the ActA and IntB, and the subsequent addition of an OVA expressing cassette under the control of the LLO promoter. Other strains of *Listeria* utilized the same OVA expression cassette, but contained different gene knockouts. For experiments on the B10.D2 background, attenuated *Listeria* expressing the HA peptide specific for $CD8^+$ T cell responses (IYSTVASSL) were used, and driven under the LLO promoter. *Listeria* were grown in brain-heart infusion media and harvested mid-log phase growth, purified by standard methods, and stored at a concentration of $>1x10^{10}$ CFU per mL at -80°C as single use stocks. Vaccinia Virus expressing the target antigens HA or OVA were expanded on Hu-

TK$^-$ cells, concentrated via ultracentrifugation, and titered by a plague assay on BSC-1 cells. *Vaccinia* viral particles were then frozen at a concentration of $5x10^6$ pfu per mL, and stored at -80°C as single use stocks.

Flow Cytometry Antibodies

Antibodies used in these studies include: CD8 Pacific Orange – Clone 5H10 (Invitrogen, Grand Island, New York), Ly6G Brilliant Violet 421 – Clone 1A8 (Biolegend, San Diego, CA), LAG-3 eflour 450 – Clone C9B7W (Ebioscience, San Diego, CA), CD45.2 Pacific Blue – Clone 104 (Biolegend, San Diego, CA), CD49b Alexaflour 488 – Clone DX5 (Biolegend, San Diego, CA), CD69 FITC – Clone H1.2F3 (BD Pharmigen, San Jose, CA), Thy1.2 FITC – Clone 53-2.1 (BD Pharmigen, San Jose, CA), TNFα eflour450 – Clone MP6-XT22 (Ebioscience, San Diego, CA), CD19 PE - Clone eBio1D3 (Ebioscience, San Diego, CA), granzyme B – Clone 16G6 (Ebioscience, San Diego, CA), Egr2 PE – Clone erongr2 (Ebioscience, San Diego, CA), Ly6C PerCp/Cy5.5 – Clone HK1.4 (Biolegend, San Diego, CA), CD160 PerCp/Cy5.5– Clone CNX46-3 (BD Pharmigen, San Jose, CA), PD-1 Pe-Cy7 – Clone J43 (Ebioscience, San Diego, CA), Tbet Pe-Cy7 – Clone eBio4B10 (Ebioscience, San Diego, CA), IFNγ Pe-Cy7 – Clone XMG1.2 (Biolegend, San Diego, CA), CD11c APC – Clone HL3 (BD Pharmigen, San Jose, CA), IL-2 APC – Clone JES6-5H4 (BD Pharmigen, San Jose, CA), Thy1.1 APC – Clone HIS51 (Ebioscience, San Diego, CA) CD11b AF700 – Clone M1/70 (Biolegend, San Diego, CA), and CD3ε APC-Cy7 – Clone 145-2C11 (Biolegend, San Diego, CA).

Extracellular Staining and FACS sorting

Cell staining was done as previously reported. In brief, prior to staining, $CD8^+$ cells were enriched using magnetic bead enrichment prior to staining. About $5x10^6$ $CD8^+$ cells were resuspended directly *ex vivo* in MACS buffer in a 96 well plate, before being resuspended in a 50μL of a mix of MACS buffer containing either the combination of antibodies or single stain controls for compensation. Cells were incubated at room temperature for 15 minutes in the dark, before excess antibody was washed off with MACS buffer. If cells were not being intracellularly stained, they were resuspended in MACs buffer and run fresh on a FacsCaliber or LSRII flow cytometer (BD, San Diego, CA). For FACS sorting, cells were stained sterilely as above, but were then sorted on a FacsAria (BD, San Diego, CA) into complete RPMI media. Following cell sorting, cells were recounted before being used in further experiments.

Intracellular Staining

For intracellular cytokine staining, cells were restimulated *in vitro* for 5 hours at 37°C with 10μM OVA (SIINFEKL) or 10μM HA (IYSTVASSL) peptide in the presence of protein transport inhibitor cocktail (Ebioscience). After five hours, cells were washed with MACs buffer, and stained extracellularly (see above). Following the extracellular stain, cells were fixed overnight using 100μL Fix/Perm buffer. The following day, cells were washed in Perm buffer, and resuspended in a 50μL of a mix of Perm buffer containing either the combination of antibodies or single stain controls for compensation. Cells were incubated at room temperature for 20 minutes in the dark, before excess antibody was washed off with Perm buffer. Cells were then resuspended in MACs buffer

and run on a FacsCaliber or LSRII flow cytometer (BD, San Diego, CA). For transcription factor staining, cells were not restimulated for 5 hours, but were instead stained directly *ex vivo*.

Data Analysis and Statistics

All data presented, with the exception of pie charts, were generated using FlowJo software (Treestar, Ashland, OR) or Graphpad Prism software, version 5.0c (GraphPad Software, La Jolla, CA). Pie charts presented were generated using Microsoft Excel 2011. Summary statistics are all presented as the mean +/- the standard error of the mean. Group means were compared using two sample student T tests, while event free survival was examined with the Kaplan-Meir method. All p-values are two-sided, and statistical significance was represented as follows; $P>0.05$ = *, $P>0.01$ = **, $P>0.001$ = ***.

CHAPTER III

LISTERIA MONOCYTOGENES BASED VACCINES PROVIDE FOR INCREASED ANTI-TUMOR IMMUNITY IN A $CD8^+$ T CELL DEPENDENT MANNER

Introduction

The generation of a potent, specific immune response to cancer is one of the driving goals of research in the field of Immunology. To this end, a variety of cancer vaccine vectors have been developed, as described above, in the hopes that a vaccine will re-start the immune system, and allow, ultimately, for tumor rejection and the generation of lifetime protective immunity. However, as several platforms of live-attenuated vaccines have been generated, it has become clear that some platforms result in better tumor rejection than others. The reason for these different efficacies is not particularly well understood on the cellular and molecular levels. Why some vaccines generate more potent $CD8^+$ T cell responses than others is a question that will require further investigation, and is the subject of this research. In this chapter, we will introduce the difference in anti-tumor efficacy generated by two different vaccine vectors; *Listeria Monocytogenes* and Vaccinia Virus. Both of these vectors are live-attenuated vaccines, which have been genetically engineered to express ovalbumin as an example tumor associated antigen. In the case of *Listeria*, the genes IntB and ActA were genetically deleted prior to use as a vaccine.

Chapter Specific Materials and Methods

Tumor Growth Experiments

Tumor implantation experiments all followed the same schedule. On day 0, 1-1.5x10^5 B16-OVA or EL4-OVA cells were implanted subcutaneously on the right flank of host mice. On day 3, host mice received intravenous vaccinations via tail vein injections of PBS, 1x10^7 cfu *Listeria-OVA* cfu, or 1x10^6 pfu Vaccinia-OVA. All

vaccinations were given in 200μL PBS. Tumor growth was measured as previously described using engineering calipers[45]. Briefly, tumors were measured every 2-3 days, and tumor size was reported as volume using the formula tumor volume=m_1 x m_2 x (π/6). For metastatic lung burden models, 1-1.5x10^5 B16-OVA cells were filtered through a 70μm nylon mesh filter before being injected via tail vein injection in 200μL PBS. On day 3, mice were treated in the same manner as the subcutaneous model. For measurement of metastatic burden, host animals were sacrificed on day 18-20, and lungs were fixed in Fekete's Solution overnight. After fixation, lungs were transferred to PBS, and spots were counted using a dissecting microscope. For survival experiments, animal survival was monitored following vaccination, and animals were euthanized if they displayed hunched behavior along with ruffled fur and heavy breathing.

$CD8^+$ T Cell Depletion

Depletion of CD8+ T cells was achieved by repeated intraperitonial administration of 200μg of 2.43 antibody (Harlan Laboratory, Dublin, VA). Mice received depleting antibody on Days -7, -5, -3, -1, 2, 7, and every 7 days to maintain depletion. On day -4, peripheral blood from depleted or undepleted mice was checked for the presence of $CD8^+$ T cells by flow cytometry. Tumor growth experiments were repeated as previously, with depleted animals receiving i.v. tumor on Day 0 and vaccination on Day 3. Survival was monitored over time.

Results

Listeria Based Vaccination Provides Superior Anti-Tumor Immunity in Both Subcutaneous and Metastatic Models

To test the hypothesis that vaccinations can provide anti-tumor immunity, we utilized a model antigen system that would allow us to examine the effects of our vaccinations in an antigen specific manner. Here, the expression of OVA by cancer cells is intended to model a unique tumor antigen, like a mutated protein expressed solely in tumor tissue. Significant research has been done to identify a variety of unique tumor antigens[146], since these antigens represent targets that the host will not tolerize responses to through normal peripheral tolerance mechanisms. Additionally, by targeting unique tumor antigens, it is possible to separate anti-tumor immunity from the development of autoimmunity. After receiving subcutaneous implantation of either B16-OVA (Fig 3-1a) or EL4-OVA (Fig 3-1b) on day 0, host mice were vaccinated on day 3 with either *Listeria*-OVA or Vaccinia-OVA intravenously, and tumor growth was measured over time. While both vaccinations did delay tumor growth, *Listeria*-OVA provided superior reduction of tumor growth in both models. Furthermore, no adoptive transfer was utilized, suggesting that both vaccines are capable of generating an anti-tumor immune response from the host's endogenous cell population.

To further examine the potential for these vaccines, we proceeded to test their efficacy in a metastatic model by giving B16-OVA intravenously on day 0 instead of subcutaneously. In this model, the lungs provide a good readout for overall tumor burden. As was seen in the flank models, Vaccinia-OVA did generate an anti-tumor effect,

resulting in decreased size and number of metastatic spots on the lungs. However, the *Listeria*-OVA vaccinated animals had significantly lower tumor burden, though some micro-metastases could still be detected under a dissecting microscope (Fig 3-2b, c). The significant decrease in tumor burden was reflected in the overall survival of these animals, as *Listeria*-OVA vaccination showed the greatest increase in median survival (Fig 3-2a). Together, these data demonstrate that the *Listeria* vaccine vector was more potent in the generation of anti-tumor immunity, despite targeting the same antigen as the Vaccinia vaccine.

Protective Immunity Generated by Either Vaccination is Dependent on the Generation of an Adaptive Immune Response

Since vaccinations can be effective in reducing tumor burden either through oncolytic mechanisms or through the generation of an immune response, we wanted to test the hypothesis that the anti-tumor response seen as a result of these vaccines was dependent on the adaptive immune system. To explore this question, we injected $RAG2^{-/-}$ mice with B16-OVA intravenously, and then proceeded to vaccinate them with PBS, *Listeria*-OVA, or Vaccinia-OVA, and examined their overall survival. In these animals, loss of RAG2 results in the absence of B cells, T cells, and NKT cells, thus no adaptive immunity can be developed. Surprisingly, the Vaccinia-OVA vaccinated animals showed a decrease in overall survival as seen in Figure 3-3. Upon closer examination, Vaccinia vaccinated mice had symptoms consistent with fatal Vaccinia challenge, including skin sores and tail decay, highlighting some of the potential risks of a live-attenuated vaccine. In agreement with a role for adaptive immunity in generating the protective response seen earlier, $RAG2^{-/-}$ animals receiving either *Listeria*-OVA or PBS had no difference in

overall median survival (Fig 3-3). These data demonstrate that neither of these vaccines have potent oncolytic function, and instead function through the generation of an adaptive immune response to produce anti-tumor effects.

Protective Immunity Generated by Listeria-OVA Vaccines is Dependent on $CD8^+$ T Cells

$RAG2^{-/-}$ animals are missing several populations of adaptive immune cells, including B cells, $CD4^+$ T cells, $CD8^+$ T cells, and NKT cells. To test if a $CD8^+$ T cell response was required for anti-tumor immunity, we utilized a depleting antibody schedule to remove only $CD8^+$ T cells from the host animals, and repeated our vaccination studies (Fig 3-4a). The antibody depletion regimen was very effective, resulting in a decreased of >99% of normal, circulating $CD8^+$ T cells after as little as two doses (Fig 3-4b,c). As can be seen in Figure 3-4d, depletion of $CD8^+$ T cells from the host mice abrogates the previous protection seen with *Listeria*-OVA vaccination. Furthermore, when $CD8^+$ T cells are depleted, *Listeria*-OVA and Vaccinia-OVA vaccinations provided similar protection, suggesting that the $CD8^+$ T cell response generated by these two vaccines was one of the key cellular differences. Interestingly, despite generating an OVA specific $CD8^+$ T cell response (Fig5-6a), Vaccinia-OVA vaccination was relatively unaffected by $CD8^+$ T cell depletion. Hence, the protection being generated by Vaccinia-OVA is predominantly not $CD8^+$ T cell dependent, and must be generating an anti-tumor adaptive immune response with some other population, likely $CD4^+$ T cells.

Summary

In summary, both *Listeria* and Vaccinia vaccination platforms resulted in anti-tumor immune responses, in an adaptive immunity based manner. This anti-tumor

immunity was generated against a variety of model tumors, and *Listeria*-OVA provided superior anti-tumor responses across all models. Interestingly, in the absence of an adaptive immune response, Vaccinia-OVA vaccination actually decreased overall survival, while *Listeria*-OVA simply offered no additional benefit. This finding shows some of the dangers of the use of live-attenuated vaccinations in certain populations, specifically those who are immunocompromised. Virulence of strains utilized as live-attenuated vaccines must be closely monitored, as many cancer patients have immune systems that are weakened by their chronic disease. Furthermore, while depletion of $CD8^+$ T cells resulted in a loss of the protective immunity generated by *Listeria*-OVA vaccination, Vaccinia-OVA protection was similar with and without depletion, suggesting that despite the generation of OVA specific $CD8^+$ T cells, these cells were non-functional, unlike those generated by *Listeria*-OVA vaccination. These data indicate that these two vaccine vectors program very different $CD8^+$ T cell responses, and that the type of response generated should be considered when designing a vector for anti-tumor immunity.

Figure 3-1

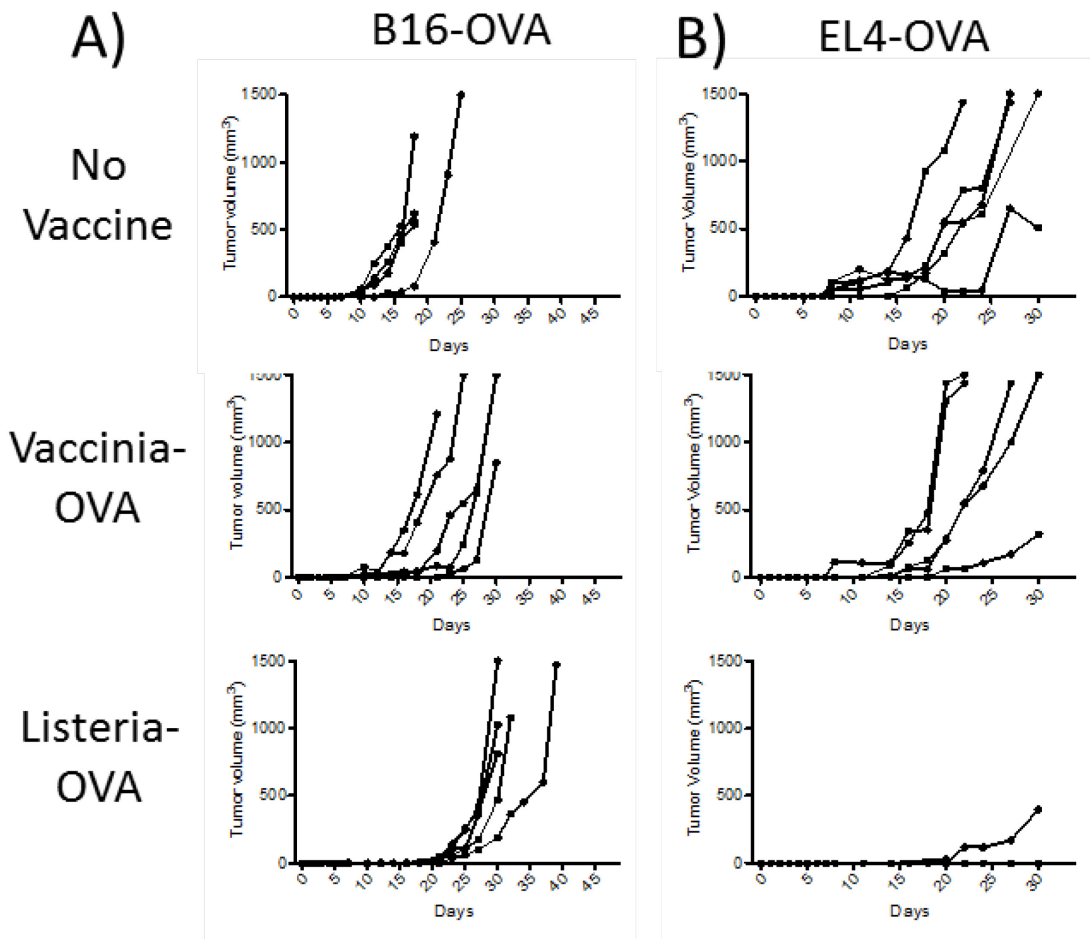
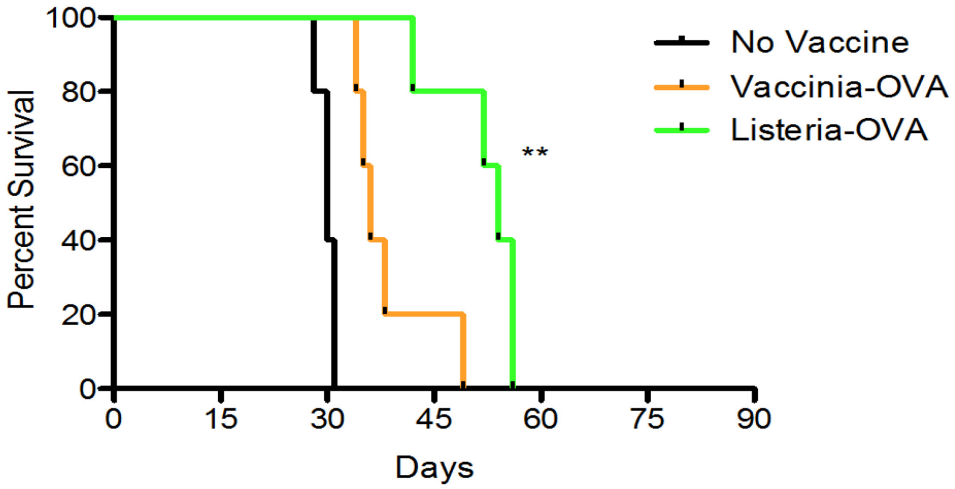


Figure 3-1: *Listeria Monocytogenes* Vaccination Produces Superior Anti-Tumor Effect in Subcutaneous Cancer Models

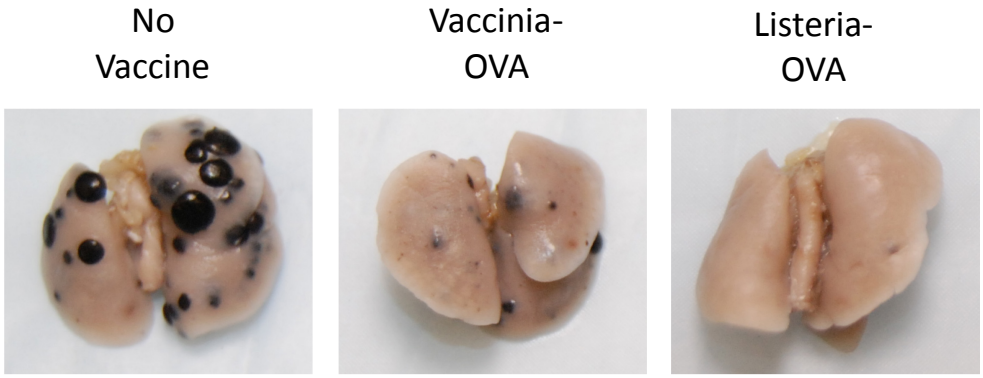
Host mice were injected subcutaneously with 1-1.5x10^5 A) B16-OVA or B) EL4-OVA on the right flank. Three days later, mice were vaccinated with either PBS, 107 cfu of *Listeria*-OVA, or 10^6 pfu of Vaccinia-OVA via intravenous injection and tumor volume was measured over time. Data shown are representative of greater than 3 independent experiments, with n=5 mice per group.

Figure 3-2

A)



B)



C)

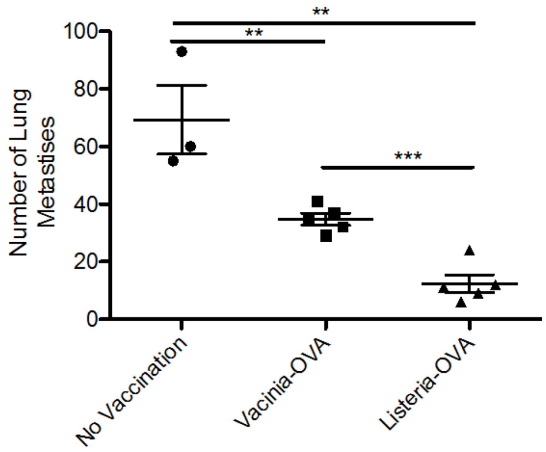


Figure 3-2: *Listeria Monocytogenes* Vaccination Produces Superior Anti-Tumor Effect in Metastatic Cancer Models

Host mice were injected intravenously with 1-1.5x10^5 B16-OVA. Three days later, mice were vaccinated with either PBS, 10^7 cfu of *Listeria*-OVA, or 10^6 pfu of Vaccinia-OVA via intravenous injection. A) Survival curves of mice receiving the individual vaccinations. B) On day 18, lungs were harvested from vaccinated animals, fixed overnight, and photographed. C) Number of micro-metastatic B16-OVA lesions counted. Data shown are representative of 3 independent experiments, with n=4-5 mice per group.

Figure 3-3

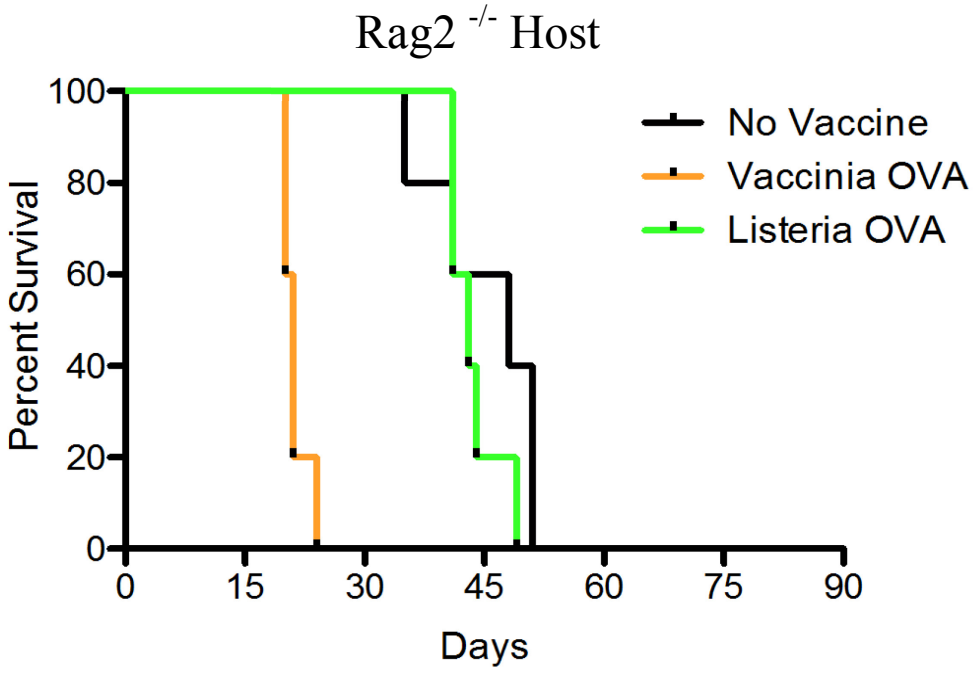
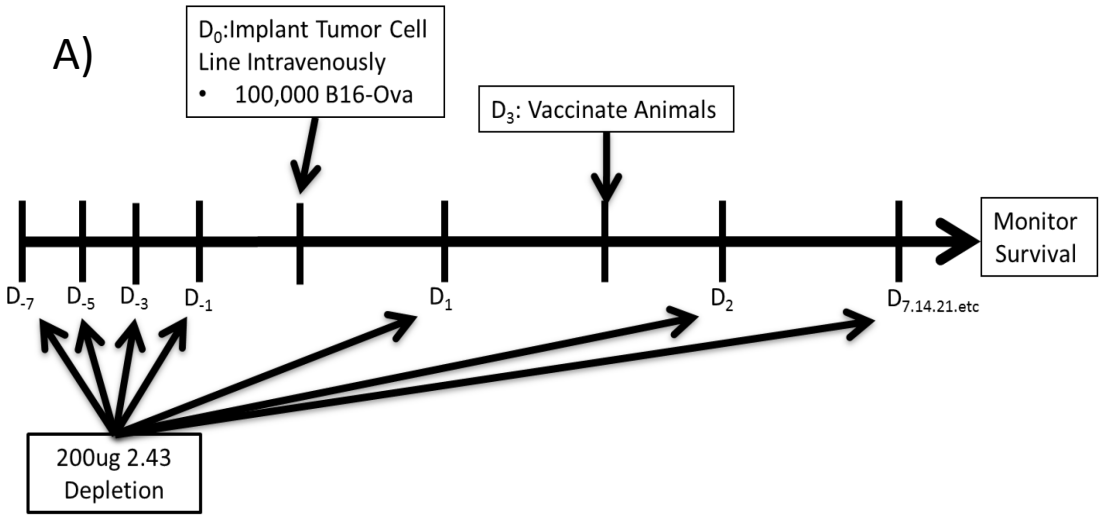
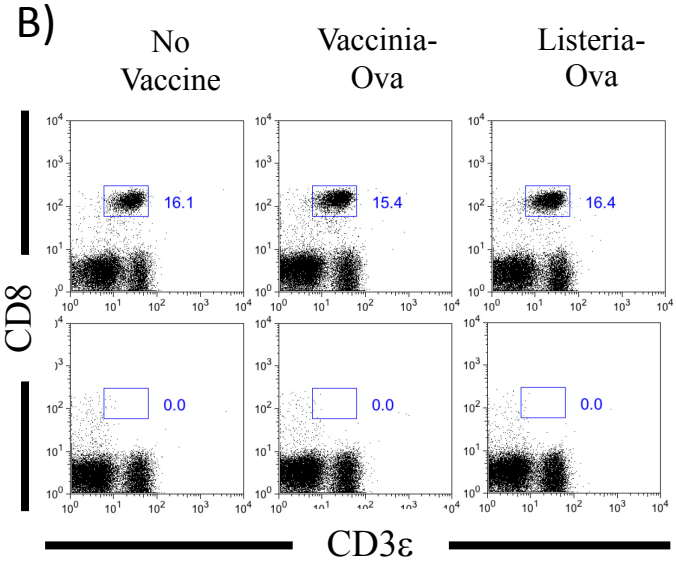


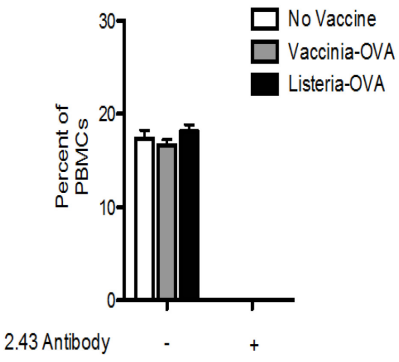
Figure 3-3: The Protective Immunity Generated by *Listeria Monocytogenes* Vaccination is Dependent Upon an Adaptive Immune Response

RAG2$^{-/-}$ host mice were injected intravenously with 1-1.5x10^{5} B16-OVA. Three days later, mice were vaccinated with either PBS, 10^{7} cfu of *Listeria*-OVA, or 10^{6} pfu of Vaccinia-OVA via intravenous injection and monitored for survival over time. Data shown are representative of 2 independent experiments, with n=5 mice per group.

Figure 3-4


A)
D0:Implant Tumor Cell Line Intravenously
• 100,000 B16-Ova
D3: Vaccinate Animals
Monitor Survival
D-7
D-5
D-3
D-1
D1
D2
D7.14.21.etc
200ug 2.43 Depletion


B)
No Vaccine
Vaccinia-Ova
Listeria-Ova
CD8
CD3ε
16.1
15.4
16.4
0.0
0.0
0.0


C)
No Vaccine
Vaccinia-OVA
Listeria-OVA
Percent of PBMCs
30
20
10
0
2.43 Antibody
-
+

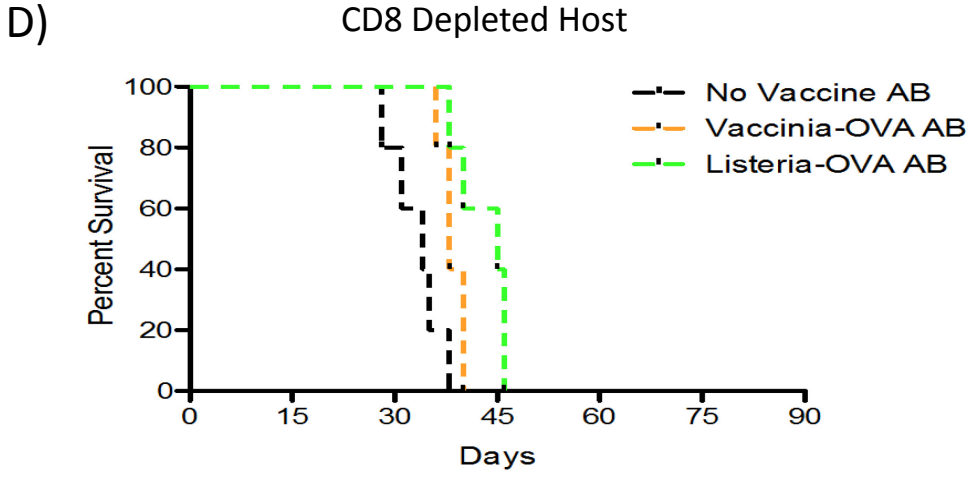

D)
CD8 Depleted Host
No Vaccine AB
Vaccinia-OVA AB
Listeria-OVA AB
Percent Survival
100
80
60
40
20
0
0
15
30
45
60
75
90
Days

Figure 3-4: The Protective Immunity Generated by *Listeria Monocytogenes* Vaccination is Dependent Upon $CD8^+$ T Cells

Prior to implanting tumor, mice were depleted of $CD8^+$ T cells by repeated intraperitonial administration of 200μg of 2.43 depleting antibody. On day zero, host mice were injected intravenously with 1-1.5x10^5 B16-OVA. Three days later, mice were vaccinated with either PBS, 10^7 cfu of *Listeria*-OVA, or 10^6 pfu of Vaccinia-OVA via intravenous injection. A) $CD8^+$ T cell depletion regimen and experimental design B) Flow cytometric analysis and C) quantification of peripheral blood $CD8^+$ T cells on day -1. D) Survival of animals over time following tumor implantation and vaccination. Data shown are representative of 2 independent experiments, with n=5 mice per group.

CHAPTER IV

LISTERIA MONOCYTOGENES BASED VACCINES RESULT IN INCREASED IN POLYFUNCTIONAL CYTOKINE PRODUCTION AND A TRANSCRIPTION FACTOR PROFILE CONSISTENT WITH ROBUST T CELL ACTIVATION

Introduction

While early studies in Immunology focused on the production of certain or single effector cytokines, more recent work has begun to identify an important role for the production of multiple cytokines. In several studies, combinatorial checkpoint protein blockade allowed for recovery of not only IFNγ, but also of TNFα and IL-2[43,45,147]. Furthermore, the generation of polyfunctional cytokine producing $CD8^+$ T cells has been linked to anti-tumor immunity. Along the same lines, early work has determined a role for the expression of single transcription factors in T cell activation and function, but more research will need to be done to examine the effects of expressing combinations of transcription factors. Finally, several different lines of research, including our own, has suggested that the initial programming of $CD8^+$ T cells is vitally important in determining their end functionality[148–150]. For example, blockade of PD-1 during the initial priming response generates $CD8^+$ T cells with increased cytolytic potential, while blockade one day after vaccinations offers no benefit[149]. In our previous studies, we were using the host's endogenous response to examine the potential benefit of these two vaccines. However, while this model is the most like a clinical model, the generation of an endogenous response only allows for immunological examination later in the priming response, once the $CD8^+$ T cell population has expanded to detectable levels. As such, we decided to investigate the cytokine and transcription factor profile in an adoptive transfer system, where we could examine the effects of these vaccines on early $CD8^+$ T responses.

Chapter Specific Materials and Methods

Adoptive Transfer and Vaccination Experiments

To study the effects of these vaccines on early $CD8^+$ T cell programming and development, adoptive transfer studies were undertaken. Briefly, $CD8^+$ T cells were harvested from the spleen and peripheral lymph nodes of 12-16 week old female OT-1 $Rag2^{-/-}$ animals (or Clone 4 $Thy1.1^+$ animals in certain experiments), passed through a 100μM cell filter, and RBCs were lysed in ACK buffer (Life Technologies, Grand Island, New York) before the remaining cells were quenched with MACS buffer and counted. Following the generation of a single cell suspension, $CD8^+$ T cells were isolated according to manufacturer's protocol utilizing a Miltenyi $CD8^+$ T cell positive isolation kit (Miltenyi, San Diego, CA). Cells were then labeled with 5μM Carboxyfluorescein Succinimidyl Ester(CFSE). For CFSE labeling, 5mM CFSE (Life Technologies, Grand Island, New York) was generated by dilution of lyophilized CFSE in DMSO, and a 5μM generated for staining by dilution in PBS. Cells were washed in cold PBS before being resuspended in a 5μM CSFE solution at a concentration of $2.5x10^7$ cells/mL. This staining solution was incubated for 8 minutes shaking at 800RPM at 37°C, before the reaction was quenched with 10X volume of ice cold, complete RPMI for 3 minutes. Cells were washed twice with ice cold PBS before being recounted, and resuspended for adoptive transfer. For adoptive transfer studies, $1\text{-}2x10^6$ $CD8^+$ T cells were admixed with their vaccination (PBS, 10^6 pfu *Vaccinia*, 10^7 cfu *Listeria*), for a final injection volume of 200 μL of cells + vaccine in PBS. Admixed cell and vaccine combos were then adoptively transferred via a single tail vein injection.

Results

$CD8^+$ T cells Responding to Listeria-OVA Vaccination Display Increased Polyfunctional Cytokine Production

To test the hypothesis that *Listeria*-OVA based vaccinations result in increased polyfunctional cytokine production in specifically activated $CD8^+$ T cells, we adoptively transferred congenically marked, CFSE labeled OVA specific $CD8^+$ T cells, named OT-1s, into host mice along with vaccinations of PBS, *Listeria*-OVA, or Vaccinia-OVA. In the interest of having a negative control for activation, we included a group of host mice that express OVA as a self-protein under the β-actin promoter. These mice, named CAG-OVA, provide a system where OT-1s recognize their cognate antigen in a non-activating condition, and will therefore be tolerized. As expected, adoptively transferred OT-1s in mice with no vaccine and no endogenous OVA expression did not divide, and produced no cytokines upon restimulation (Fig 4-1a,b). In contrast, OT-1s exposed to the tolerizing conditions of the CAG-OVA host did divide, but produced very few cytokines. Vaccinia-OVA vaccinated OT-1s produced all of the cytokines examined, including IFNγ, TNFα, IL-2, and Granzyme B. However, *Listeria*-OVA primed OT-1s produced the largest populations of cytokine producing cells for all the cytokines examined, with the notable exception of IL-2 (Fig4-1a, b). Interestingly, Vaccinia-OVA primed OT-1s produced the most $IL\text{-}2^+$ cells, suggesting again that they may be programmed differently than OT-1s responding to *Listeria*-OVA. However, while there was a statistically significant increase, the percentage of OT-1s producing effector cytokines in response to *Listeria* was only about 10% higher than those responding to Vaccinia. Therefore, we found it

unlikely that this small, but statistically significant increase in cytokine production was the driving force behind the increased anti-tumor effect observed earlier.

To examine the ability of the vaccinated OT-1s to produce multiple cytokines, we quantified all possible combinations of the four individual cytokines examined. Perhaps not surprisingly, we found that *Listeria*-OVA vaccinated OT-1s had the greatest percentage of cells producing two or more cytokines (Fig 4-2a,b). Furthermore, the increase in polyfunctional $CD8^+$ T cell responses correlated with decreased percentages of OT-1s making none of the examined cytokines when compared to Vaccinia-OVA primed OT-1s (Fig 4-2). Together, these data show that as early as three days post vaccination, the majority of the cells responding to both vaccines produce multiple cytokines. Yet, Vaccinia-OVA and *Listeria*-OVA based vaccines result in different cytokine profiles within the responding $CD8^+$ T cells, with *Listeria*-OVA vaccinated $CD8^+$ T cells producing larger populations of cytokine expressing cells, as well as increased populations expressing multiple cytokines. As polyfunctional $CD8^+$ T cells have been shown to have increased anti-tumor efficacy, the differences in the cytokine profile generated in response to these two vaccines could potentially explain some of the differences in anti-tumor immunity being generated.

$CD8^+$ T cells Responding to Listeria-OVA Vaccination Display An Activation Focused Transcription Factor Profile

Given that we saw significant differences in cytokine production, we next tested the hypothesis that *Listeria*-OVA and Vaccinia-OVA generate different transcription factor profiles in responding $CD8^+$ T cells. Similar to the previous experiments, we

adoptively transferred OT-1s into the previous four conditions for three days. As seen before, in the absence of antigen, OT-1s did not divide and did not up regulate any of the transcription factors investigated. Interestingly, adoptive transfer of OT-1s into CAG-OVA hosts resulted in these cells dividing, as seen before, but they did not up regulate transcription factors previously correlated with $CD8^+$ T cell activation, namely Tbet and Eomesedermin. However, they did up regulate a transcription factor originally identified as a tolerogenic $CD4^+$ T cell transcription factor, named Egr2 (Fig 4-3a,b). The $CD4^+$ T cell literature[151,152], along with the expression of Egr2 in the OT-1s transferred into a CAG-OVA hosts, suggests that this factor is also associated with $CD8^+$ T cell tolerance, and may work against $CD8^+$ T cell activation. Surprisingly, while Vaccinia-OVA vaccination did result in up regulation of the activating transcription factors Tbet and Eomesedermin, it also resulted in the up regulation of Egr2, creating a mixed transcription factor profile. In contrast, *Listeria*-OVA based vaccination caused only unregulation of the activating factors, without up regulation of Egr2 (Fig 4-3a,b).

As before, we wanted to examine the profile of combined expression, as each transcription factor likely has input into the eventual functionality of the $CD8^+$ T cells. Interestingly, when assessing the percent of the overall $CD8^+$ OT-I T cell population expressing any of the combinations of transcription factors, CAG-OVA stimulated OT-1s expressed predominantly Egr2 alone. Vaccinia-OVA vaccinated OT-1s expressed predominantly combinations of Tbet and Eomesedermin, but about 15% of the population did express combinations of Tbet and Eomesedermin along with Egr2. About 5% of OT-1s responding to Vaccinia-OVA vaccination even expressed Egr2 alone (Fig 4-4). This is significantly different than Listeria-OVA vaccinated OT-1s, where only about 5% of the

cells expressed Egr2 in any form, and about 85% of the cells expressed only combinations of Tbet and Eomesedermin. Taken together, *Listeria*-OVA vaccinations seem to drive a more activating $CD8^+$ T cell transcription factor profile, and do not drive expression of tolerogenic factors such as Egr2. In contrast, while Vaccinia-OVA primed $CD8^+$ are activated, they may be sub-optimally activated as they up regulate expression of the tolerogenic transcription factor Egr2.

Summary

While the expression of single effector proteins has resulted in increased understanding of the roles each of those proteins play in an immune response, current research has begun to unravel the meaning behind combinations of effector cytokines, or combinations of transcription factors. In these data, we demonstrate that *Listeria*-OVA vaccines generate increases both in the percentage of $CD8^+$ T cells expressing individual effector cytokines, as well as those expressing of multiple cytokines, with the notable exception of IL-2. Furthermore, Vaccinia-OVA vaccination did generate expression of classical $CD8^+$ T cell activation transcription factors, but did not drive expression of these activation markers exclusively. Populations of cells expressing the tolerogenic transcription factor Egr2 were seen, unlike during the response to *Listeria*-OVA. The resulting transcription factor phenotype is likely the driving force behind the differences in cytokine production, as Tbet and Eomesedermin have been shown to play a prominent role in the production of effector cytokines during $CD8^+$ T cell activation[153,154]. Interestingly, in Tbet knockout $CD8^+$ T cells, increases in IL-2 production have been seen, correlating with our data on *Listeria* and Vaccinia vaccinations[154]. Furthermore, Egr2 knockout CD4 T cells produce more IFNγ when stimulated both *in vitro* and *in*

vivo[155], suggesting that the mixed expression of Tbet, Eomesedermin, and Egr2 in $CD8^+$ T cells responding to Vaccinia may correlate with the lower percentages of cytokine producing cells. However, these differences, while statistically significant, biologically seem small in scale, and so are unlikely, in our opinion, to be the driving molecular difference between the functionality of the $CD8^+$ T cell response generated by these two vaccines. Nevertheless, these data do support the hypothesis that these vaccines program a fundamentally different $CD8^+$ T cell response, and that this difference is clear as early as three days following vaccination.

Figure 4-1

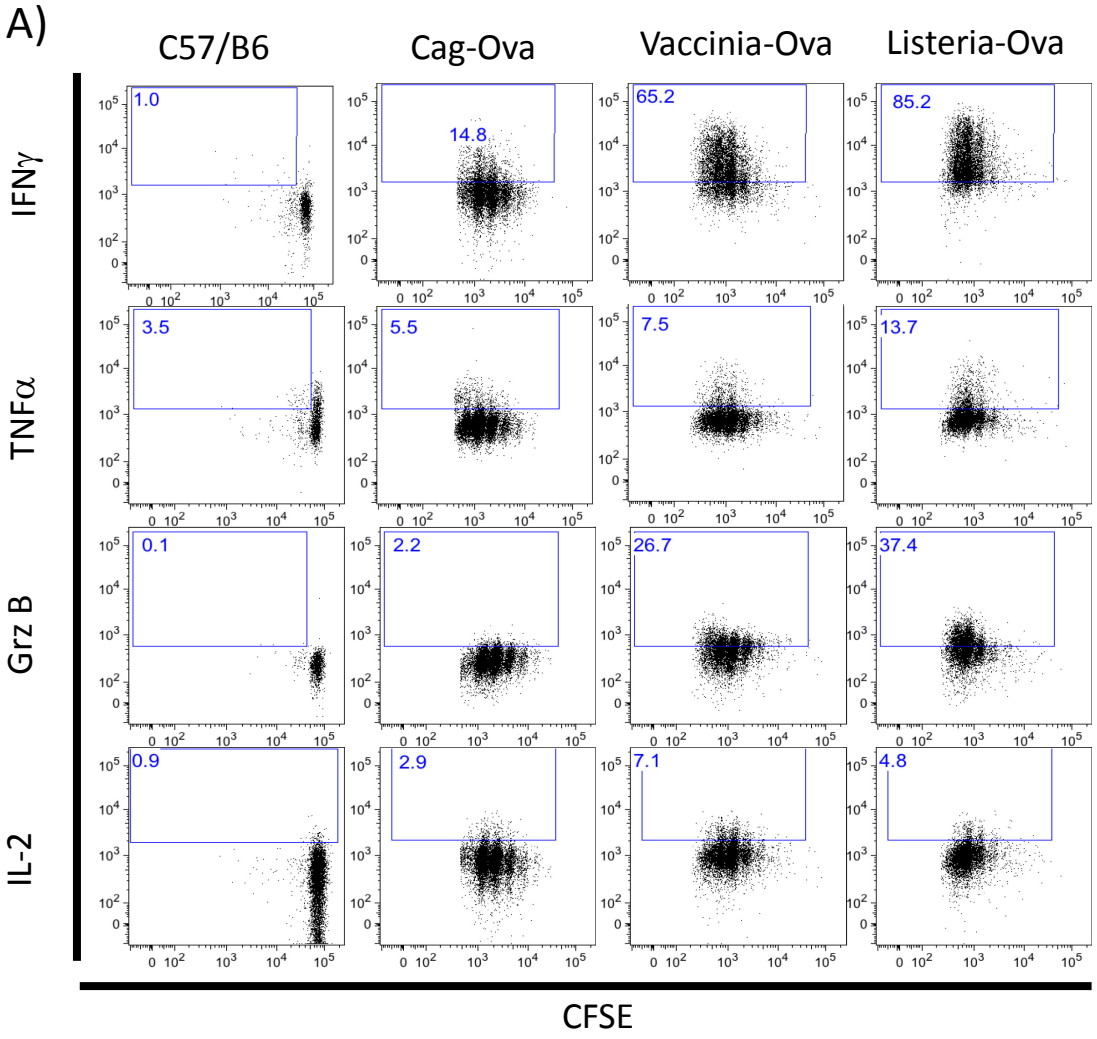
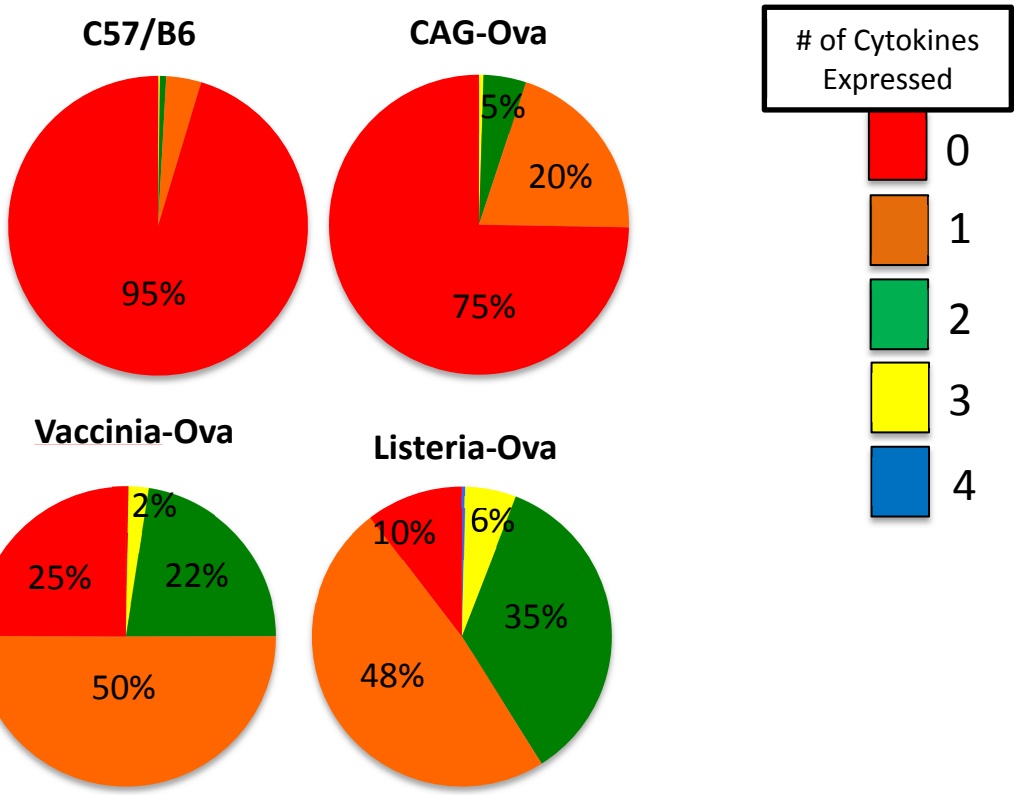

A)
C57/B6
Cag-Ova
Vaccinia-Ova
Listeria-Ova
IFNγ
TNFα
Grz B
IL-2
1.0
14.8
65.2
85.2
3.5
5.5
7.5
13.7
0.1
2.2
26.7
37.4
0.9
2.9
7.1
4.8
CFSE
B)
Percent Of Divided Cells Expressing Granzyme B
Percent Of Divided Cells Expressing TNFa
Percent Of Divided Cells Expressing IL2
C57/B6 Host
Cag-Ova
Vacc-Ova
Listeria-Ova

Figure 4-1: *Listeria Monocytogenes* Vaccinations Result in Larger Populations of Effector Cytokine Producing Cells

CFSE labeled OT-1s were admixed with their vaccine and then adoptively transferred into recipient host mice, either congenically marked CD45.1^{+} wildtype or CAG-OVA mice. On day three, spleens were harvested, and splenocytes were restimulated with OVA peptide in the presence of protein transport inhibitor for 5 hours prior to intracellular staining for cytokine expression. A) Cells were gated on CFSE expression as well as CD8 and CD45.2, and are depicted as A) Flow cytometry dot plots and B) quantified graphs of the percent of each population expressing a given cytokine. Data shown are representative of greater than 3 independent experiments, with n=3-4 mice per group.

Figure 4-2

A)


C57/B6
95%
CAG-Ova
5%
20%
75%
Vaccinia-Ova
2%
25%
22%
50%
Listeria-Ova
10%
6%
35%
48%
# of Cytokines Expressed
0
1
2
3
4

B)

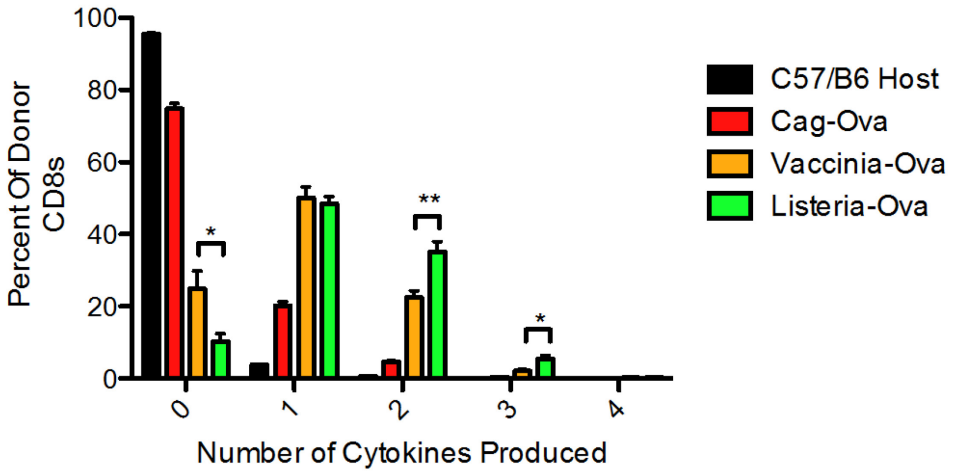

Percent Of Donor CD8s
100
80
60
40
20
0
*
**
*
0
1
2
3
4
Number of Cytokines Produced
C57/B6 Host
Cag-Ova
Vaccinia-Ova
Listeria-Ova

Figure 4-2: *Listeria Monocytogenes* Vaccinations Result in Larger Populations of Polyfunctional $CD8^+$ T Cells

CFSE labeled OT-1s were admixed with their vaccine and then adoptively transferred into the recipient host mice as previously. A) Cells were gated on CFSE expression as well as CD8 and CD45.2, before expression of all possible combinations of cytokines were examined as A) pie charts and B) quantifiable bar charts. Data shown are representative of greater than 3 independent experiments, with n=3-4 mice per group.

Figure 4-3

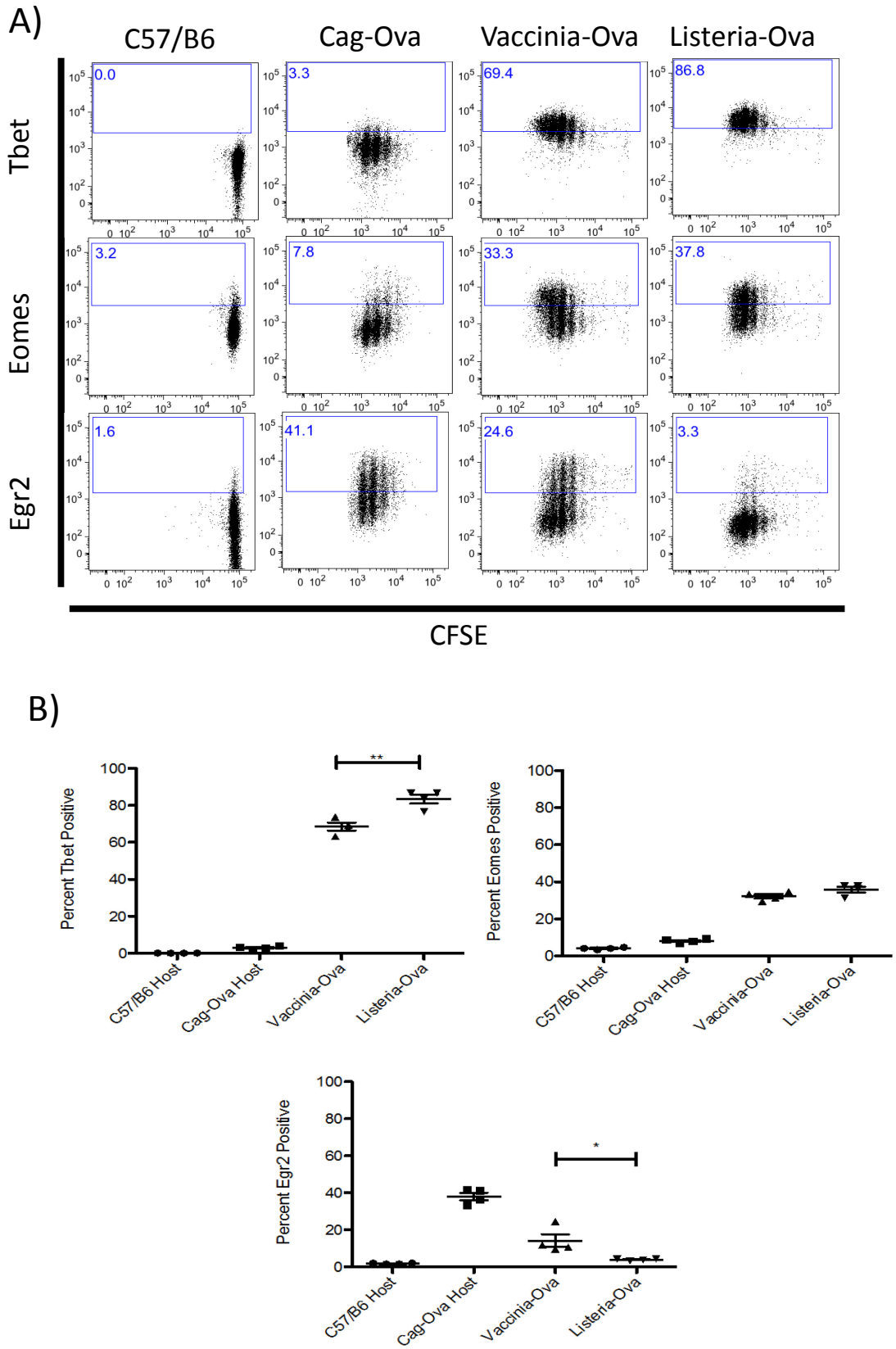

A)
C57/B6
Cag-Ova
Vaccinia-Ova
Listeria-Ova
Tbet
Eomes
Egr2
0.0
3.3
69.4
86.8
3.2
7.8
33.3
37.8
1.6
41.1
24.6
3.3
CFSE
B)
Percent Tbet Positive
Percent Eomes Positive
Percent Egr2 Positive
C57/B6 Host
Cag-Ova Host
Vaccinia-Ova
Listeria-Ova
**
*

Figure 4-3: *Listeria Monocytogenes* Vaccinations Result in Increased Populations of Cells Expressing Activating Transcription Factors

CFSE labeled OT-1s were admixed with their vaccine and then adoptively transferred into recipient host mice, either congenically marked CD45.1^{+} wildtype or CAG-OVA mice. On day three, spleens were harvested, and splenocytes stained intracellularly for transcription factor expression. A) Cells were gated on CFSE expression as well as CD8 and CD45.2, before being examined as for expression of individual transcription factors and B) quantified. Data shown are representative of greater than 3 independent experiments, with n=3-4 mice per group.

Figure 4-4

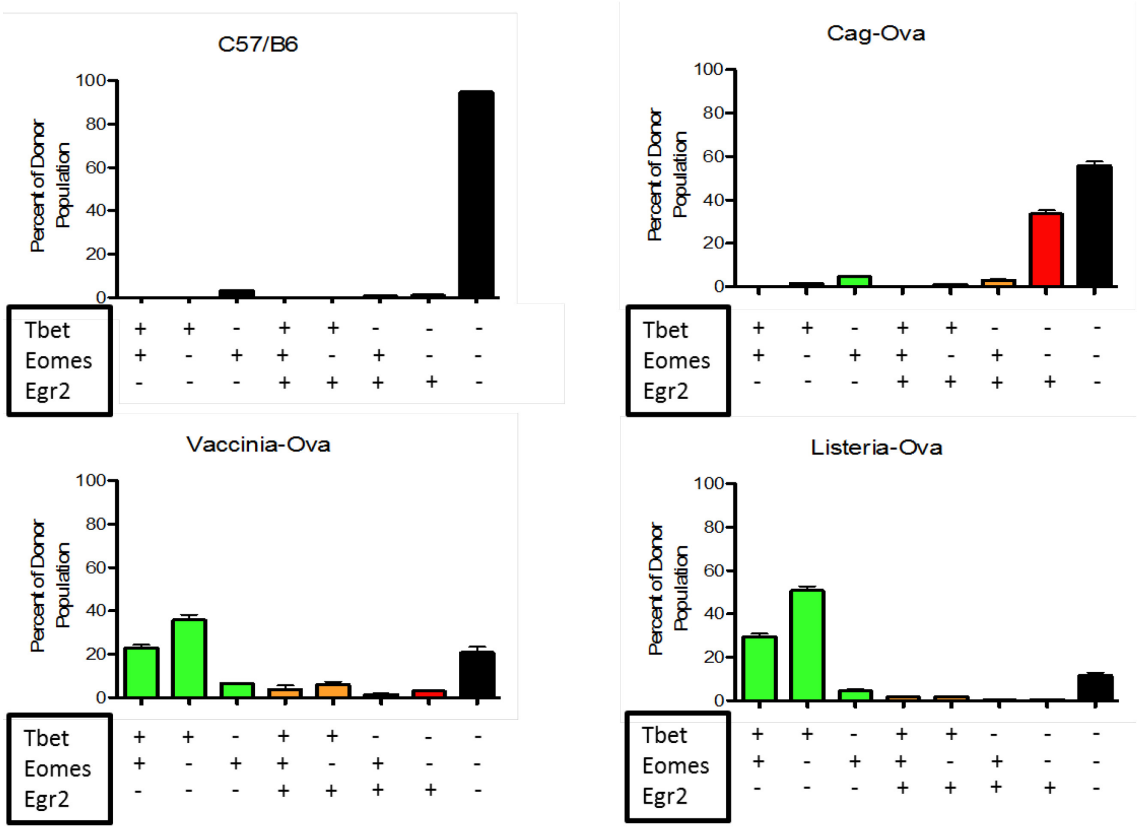


Figure 4-4: *Listeria Monocytogenes* Based Vaccinations Result in a More Activation Focused Transcription Factor Profile

CFSE labeled OT-1s were admixed with their vaccine and then adoptively transferred into the same recipient host mice as previously described. Cells were gated on CFSE expression as well as CD8 and CD45.2, before being examined as for expression of all possible combinations of transcription factors. Data shown are representative of greater than 3 independent experiments, with n=3-4 mice per group.

CHAPTER V

LISTERIA MONOCYTOGENES BASED VACCINES ALLOW FOR CD8$^+$ T CELL ACTIVATION WITHOUT THE UP REGULATION OF PD-1 OR LAG-3

Introduction

As discussed above, checkpoint proteins play an important role in maintaining tolerance to self-proteins as well as controlling the magnitude of the immune response being generated. Previous studies in our lab have demonstrated that the addition of *in vivo* PD-1[149] or LAG-3[44] blockade during activation in the settings of self-tolerance or infection results in increased magnitude of T cell expansion as well as increased cytokine production. Blockade of PD-1 during the course of an acute infection plays a role in the eventual development of memory function, though whether it positively or negatively regulates the development of memory T cells has yet to be fully understood. In one study, blockade of PD-1 during the course of Vaccinia infection resulted in increased acute and memory responses, as well as increased expression of central memory markers like CCR7, as well as increased IL-2 production[156]. Additionally, in another study, PD-1 knockout animals were able to survive a dose of the fungus *Histoplasma Capsulatum* that was 10x the normal lethal dose, suggesting that PD-1 expression negatively regulates the development of host immunity during acute infections[157]. Likewise, blockade of LAG-3 has been found to expand T cell response to a variety of stimuli[27]. Additionally, combinatorial blockade of multiple checkpoint proteins has been found to generate potent responses to typically chronic infection[33,147,158]. However, the fact that blockade of checkpoint proteins assists in the generation of an acute response to vaccination is predicated on the expression of these checkpoints during the activation of the adaptive immune response. Indeed, checkpoint protein expression has long been a hallmark of $CD4^+$ and $CD8^+$ T cell activation. However, the exact expression of certain checkpoints in response to individual vaccines has not been fully explored, and future research is

necessary to determine if certain combinations of checkpoint proteins are expressed preferentially in the acute settings of vaccination.

Chapter Specific Materials and Methods

Tetramer Staining and Memory Rechallenge Experiments

To stain tetramer-specific cells, we modified the previous extracellular staining protocol to include a new initial step. For staining tetramer positive cells in the peripheral blood, approximately 100 μL of blood was collected into 2% sodium citrate in complete RPMI from a small incision in the tail vein daily. Following collection of the blood, styptic powder (Petco, Baltimore, MD) was used to stop the tail from bleeding. RBCs were lysed for 5 minutes in 200 μL of ACK buffer, before being washed twice with 200μL MACs buffer. For some studies, single cell suspensions from the spleen or lymph nodes were stained as well. To stain tetramer, once a single cell suspension had been obtained, cells were incubated for 25 minutes at room temperature in the dark with OVA tetramer reagent (Beckman Coulter, Brea, CA) resuspended in MACs buffer, at a concentration of about $5x10^6$ per 50 μL. Cells were washed once with 200 μL MACS buffer, before extracellular staining was done as previously stated.

For memory experiments, host mice were primed on Day 0 with either PBS, 10^6 pfu Vaccinia-OVA, or 10^7 cfu *Listeria*-OVA, all by tail vein injection. On Day 40, mice were rechallenged subcutaneously on Day 40 with an emulsion of Addavax (Invivogen, San Diego, CA) containing 10 μg of whole OVA protein. For a control for tetramer staining, some mice received 10^7 cfu *Listeria-OVA* via tail vein injection on Day 40. On Day 45, spleens and draining lymph nodes were harvested to examine the effects of a

Listeria or Vaccinia based prime on subsequent recall responses as well as PD-1 and LAG-3 expression.

Results

Listeria Based Vaccinations Drive CD8$^+$ T Cell Divisions Without the Up Regulation of PD-1 or LAG-3

The role of checkpoint proteins in the development of an immune response has been documented in a variety of models. Therefore, we investigated whether our vaccinations caused different expression of checkpoint proteins during the early stages of CD8$^+$ T cell activation. Similar to previous experiments, we chose to investigate this question through the use of an adoptive transfer model. As anticipated, adoptive transfer of OT-1s into a tolerizing host (CAG-OVA) resulted in robust up regulation of both PD-1 and LAG-3. As has been seen in other models CD8$^+$ T cell activation, Vaccinia-OVA vaccine caused intermediate up regulation of both PD-1 and LAG-3 on the adoptively transferred OT-1s, as well as some expression of 2B4. Surprisingly, however, *Listeria*-OVA primed OT-1s showed no up regulation of PD-1, and minimal expression of LAG-3 (Fig 5-1a). This was confirmed by adoptively transferring PD-1$^{-/-}$ OT-1s into the same host groups, and comparing the expression of PD-1 on wildtype OT-1s to that of PD-1$^{-/-}$ OT-1s when both when given *Listeria*-OVA as a vaccination (Fig 5-2a,b). To analyze this phenotype deeper, we examined the mean fluorescence intensity (MFI) of PD-1 and LAG-3 on OT-1s as a function of division for our two vaccinations. The expression of PD-1 and LAG-3 on OT-1s primed against Vaccinia-OVA matched the expression profile reported in other vaccine studies, namely peaking on the first division, and

decreasing as the cells divided further. However, the expression of PD-1 on OT-1s responding to *Listeria*-OVA never increased, and in fact, stayed relatively stable despite multiple divisions (Fig 5-1b). LAG-3 expression also did not have the expected early peak, instead staying well below the MFI on Vaccinia-OVA vaccinated OT-1s. Interestingly, the expression of CD160 was also significantly different when examined as MFI, though the biological significance of this is currently unclear.

Listeria Based Vaccinations Drive CD8$^+$ T Cell Activation Without Combinatorial Expression of Checkpoint Proteins

Having observed such a striking phenotype when examining individual checkpoint proteins, we proceeded to analyze the combinatorial expression of these molecules. As would be expected from the individual expression data, the greatest combinatorial expression of checkpoints was seen on the OT-1s adoptively transferred into CAG-OVA hosts. Vaccinia-OVA vaccinated OT-1s also showed expression of multiple checkpoints, with approximately 25% of the population expressing 2 or more of the indicated checkpoints. However, OT-1s from *Listeria*-OVA vaccinated mice had 75% of the population expressing none of the examined checkpoints, and only about 4% expressing 2 or more checkpoints (Fig 5-3a).

As different combinations of checkpoint proteins have been reported in the literature, we also wanted to analyze specifically which combinations of checkpoints were being expressed. Interestingly, when examining the combinations seen on the OT-1s transferred into CAG-OVA hosts, the dominant populations of cells expressed PD-1 alone (~60%), followed by PD-1 and LAG-3 (~25%), suggesting that PD-1 and LAG-3

play prominent roles in early tolerance mechanisms. On Vaccinia-OVA primed OT-1s, combinations of PD-1 and LAG-3 were prevalent (~40%), while approximately 40% of the cells expressed none of the checkpoint proteins investigated (Fig 5-3b). Surprisingly, a population of cells expressing LAG-3 alone also existed, though this may be a function of vaccinations being generally activating settings, while most co-expression studies to date have been pursued in tolerizing conditions. Finally, in OT-1s being primed by *Listeria*-OVA, the vast majority of cells (~75%) expressed none of the checkpoint proteins, followed by expression of 2B4 alone. Together, these data show that *Listeria*-OVA vaccines have an extremely unique phenotype and allow for early $CD8^+$ T cell activation without expression of multiple checkpoint proteins. Furthermore, to the best of our knowledge, these data are the first to demonstrate that checkpoint protein up regulation may not be a requirement for $CD8^+$ T cell activation, but instead that CD8+ T cells up regulate certain checkpoint proteins in response to individual pathogens.

$CD8^+$T Cell Activation Without PD-1 Up Regulation is a Function of the ActA Attenuation in the Listeria Vector

As *Listeria Monocytogenes* is a relatively well studied pathogen in the field of immunology, we wanted to explore why this $CD8^+$ T cell phenotype had not been observed previously. The *Listeria* vaccine being utilized in these studies was attenuated through the genetic deletion of two genes; Internalin B (IntB) and Actin Assembly Inducing Protein A (ActA)[136]. With these genes knocked out, the *Listeria* vector tends to end up specifically in phagocytic cells of the immune system (IntB), and cannot escape the originally infected cell through the use of actin tails (ActA). Therefore, we wanted to test the hypothesis that the genetic attenuations of our strain of *Listeria* created the unique

$CD8^+$ T cell phenotype we had observed. To do this, we adoptively transferred CFSE labeled OT-1s into congenically marked host mice with either PBS, WT *Listeria*-OVA, IntB $^{-/-}$ *Listeria*-OVA, ActA $^{-/-}$ *Listeria*-OVA, or ActA $^{-/-}$ IntB $^{-/-}$ *Listeria*-OVA (the strain we used in the previous experiments). We also adoptively transferred CFSE labeled OT-1s into CAG-OVA host mice as a positive control for PD-1 up regulation. Interestingly, WT *Listeria*-OVA vaccination resulted in the adoptively transferred OT-1s up regulating PD-1 during their activation (Fig 5-4). This phenotype was mirrored when IntB $^{-/-}$ *Listeria*-OVA was used as the vaccine, suggesting that the loss of IntB is not what is causing this $CD8^+$ T cell phenotype. However, ActA $^{-/-}$ *Listeria*-OVA primed OT-1s were an exact phenocopy of the fully attenuated strain used in the previous experiments, suggesting that the deletion of ActA from the *Listeria* genome resulted in the activation of $CD8^+$ T cells without up regulation of PD-1.

$CD8^+$ T Cell Activation Without PD-1 Up Regulation is Not a Function of the OT-I Adoptive Transfer Model

Since previous reports have always seen PD-1 up regulation as a function of $CD8^+$ T cell activation, we wanted to further explore whether the unique phenotype generated by *Listeria* based vaccination was a function of the model system being utilized. To test this, we replicated our previous adoptive transfer studies utilizing a different $CD8^+$ T cell transgenic donor mouse, Clone 4, which produces $CD8^+$ T cells specific for the influenza protein hemaglutinin (HA) on a B10.D2 background (H-$2k^d$). Similar to the previous experiments, we adoptively transferred these HA specific $CD8^+$ T cells into new hosts along with PBS, Vaccinia-HA, or *Listeria*-HA. We also adoptively transferred $CD8^+$ Clone 4 T cells into a mouse model expressing HA as a self-protein,

named C3-HA^{lo}, as a model of non-inflammatory activation as well as positive control for PD-1 expression. When we examined PD-1 and LAG-3 expression on the adoptively transferred cells after three days, we found the same phenotype we discovered when using the OT-1:C57/B6 system. As before, Clone 4s transferred into C3-HA^{lo} hosts divided in response to their target antigen, but also expressed high levels of the checkpoint proteins PD-1 and LAG-3(Fig 5-5a). In mice that received Vaccinia-HA, PD-1 and LAG-3 expression on the dividing Clone 4s was intermediate, while Clone 4s primed against *Listeria*-HA expressed negligible levels of PD-1 and LAG-3. Additionally, as seen in the C57/B6 system, PD-1 and LAG-3 expression as a function of division was minor in the *Listeria*-HA vaccinated mice (Fig 5-5b). Together, these data show that activation of CD8$^+$ T cells without PD-1 expression by *Listeria* based vaccines is not a function of the model system being utilized, but is instead of function of the vaccine vector being used.

Listeria Monocytogenes Based Vaccines Result in Significantly Decreased PD-1 and LAG-3 Expression on Endogenously Generated, Vaccine Specific CD8$^+$ T cells

While our adoptive transfer system allows for examination of the earliest events of CD8$^+$ T cell activation, in our tumor treatment models, we did not utilize adoptive transfer therapy. Therefore, we examined the expression of PD-1 and LAG-3 on OVA tetramer$^+$ CD8$^+$ T cells in the peripheral blood of host animals following vaccination. As we saw previously, *Listeria*-OVA vaccination resulted in a greater expansion of tetramer$^+$ CD8$^+$ T cells (Fig 5-6a). Surprisingly, even during the generation of an endogenous CD8$^+$ T cell response, *Listeria* based vaccination resulted in significantly decreased expression of both PD-1 and LAG-3, though we did see some expression of these proteins during the

generation of the endogenous response (Fig 5-6b,c). Importantly, the largest difference between the $CD8^+$ T cell responses generated by the two vaccine vectors was the expression of PD-1 with or without LAG-3. Together, these data show that *Listeria* based vaccines result in significantly decreased PD-1 and LAG-3 expression in both adoptive transfer models, as well as in the generation of an endogenous $CD8^+$ T cell response.

Listeria Monocytogenes Vaccination Does Not Prevent PD-1 Expression During Memory Responses

Since expression of PD-1 has previously been correlated with $CD8^+$ T cell activation during vaccinations, we wanted to see if our *Listeria* primed $CD8^+$ T cells were capable of PD-1 expression during a memory response, or if PD-1 expression on these $CD8^+$ T cells had been permanently altered in some way by this vaccination. To test the hypothesis that PD-1 expression was only affected during the initial exposure to *Listeria* vaccination, mice were vaccinated with PBS, Vaccinia-OVA, or *Listeria*-OVA. Forty days after the initial vaccines, mice were rechallenged with whole OVA protein mixed with Addavax adjuvant subcutaneously. Interestingly, unlike previous reports, we found no deficit in the second expansion of tetramer positive cells in the draining lymph of mice receiving the *Listeria*-OVA priming vaccine, and in fact, found a larger population of tetramer positive cells than with in mice primed with Vaccinia-OVA (Fig 5-7a). Additionally, unlike during the primary response to these vaccines, we found no difference in either $CD8^+$ T cell expansion or checkpoint protein expression between our two vaccines (Fig. 5-7b,c). Therefore, PD-1 and LAG-3 expression is modulated during the priming response to *Listeria* based vaccination, but is not repressed permanently throughout the life of these $CD8^+$ T cells.

PD-1 Expression During Vaccination is One of the Molecular Causes of Differing Anti-Tumor Immunity

Due to the striking PD-1 expression differences between OT-1s responding to Vaccinia-OVA and those responding to *Listeria*-OVA, we wanted to test whether this difference resulted in the differences in anti-tumor immunity generated by these two vaccines. To test this hypothesis, we utilized PD-$1^{-/-}$ host mice, such that no cells could up regulate PD-1, with or without vaccination. We then repeated our prior intravenous tumor vaccination study in these new host animals, examining survival as a readout of anti-tumor immunity. Strikingly, despite the differences in anti-tumor immunity generated by these vaccines in wildtype animals, PD-$1^{-/-}$ host animals showed equal survival benefit with Vaccinia-OVA or *Listeria*-OVA vaccination (Fig 5-8), suggesting that the differences we observed in PD-1 expression were biologically significant, and potentially the main molecular difference creating the observed functional difference.

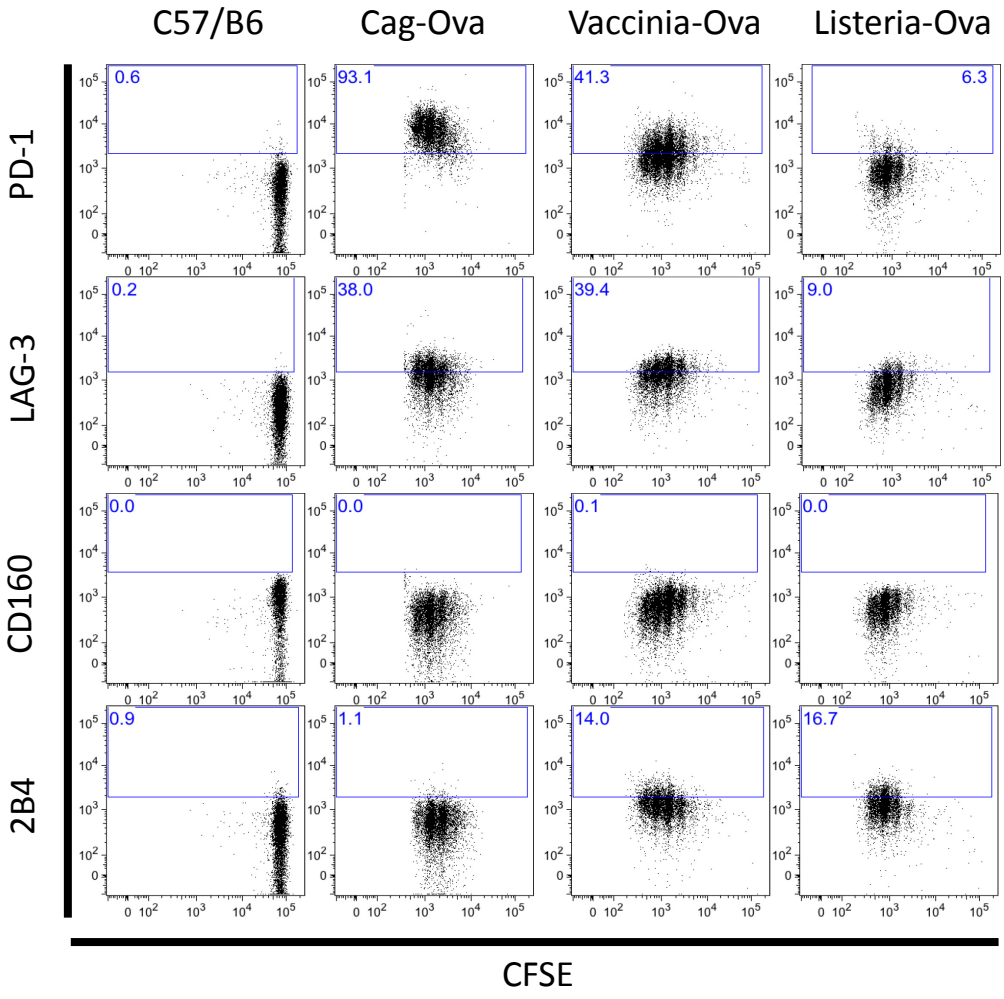
Summary

The role of checkpoint proteins in early T cell activation has just begun to be understood. While PD-1 expression has sometimes been used as a readout for $CD8^{+}$ T cell activation in vaccination settings, both our lab as well as others have shown the therapeutic effects of blocking PD-1 or LAG-3 during the early priming response, suggesting that these proteins are still signaling during activation. Checkpoint blockade during the priming phase often results in increased initial expansion, increased cytokine production, and higher levels of functional cytolytic activity. Perhaps not surprisingly, the molecular advantages we observed phenotypically when using a *Listeria* vaccination

mimic those seen during checkpoint blockade studies, further suggesting that checkpoint protein expression is one of the defining differences between these two vaccinations. Here, we have shown that checkpoint expression is not a pre-requisite for $CD8^+$ T cell activation, and in fact, some vaccine vectors may modulate expression of individual checkpoint proteins differently. Furthermore, modulation of PD-1 expression is not a function of the adoptive transfer model, or the target antigen and mouse background. This phenotype is dependent specifically on the genetic deletion of ActA from the *Listeria* vaccine, demonstrating that the key to this phenotype is likely the *Listeria* entering the cytoplasm of an APC and staying there. Finally, by deleting PD-1 and preventing either vaccine from driving PD-1 expression, we were able to extend the survival time of animals receiving Vaccinia-OVA, such that these two vaccines had similar anti-tumor effects. Thus, the difference in PD-1 expression during response to these two vaccines is likely one of the major molecular differences that explains their difference in anti-tumor efficacy.

Figure 5-1

A)


C57/B6
Cag-Ova
Vaccinia-Ova
Listeria-Ova
PD-1
LAG-3
CD160
2B4
0.6
93.1
41.3
6.3
0.2
38.0
39.4
9.0
0.0
0.0
0.1
0.0
0.9
1.1
14.0
16.7
CFSE

B)

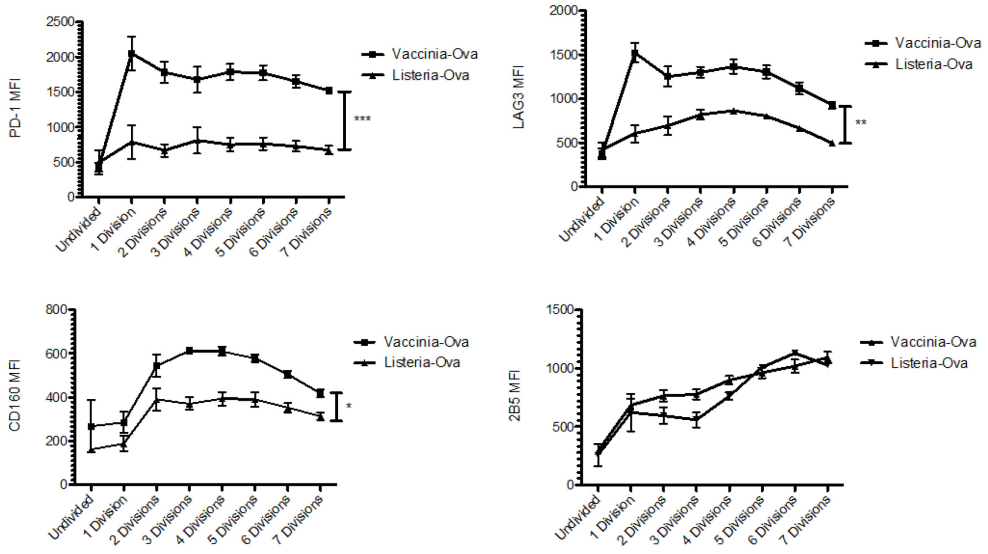
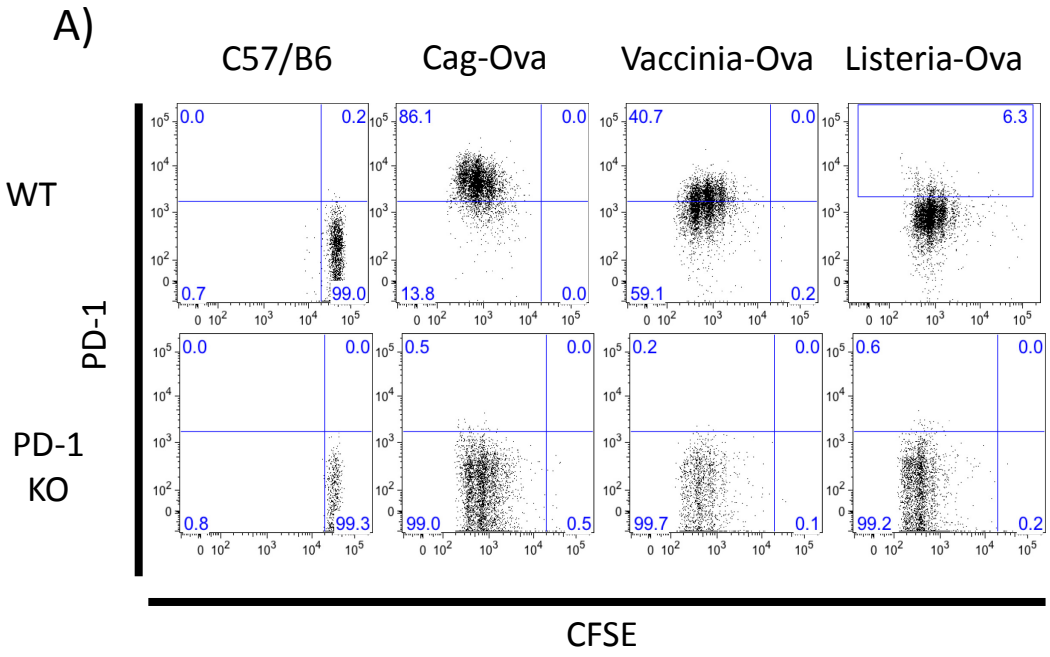

PD-1 MFI
LAG3 MFI
CD160 MFI
2B5 MFI
Vaccinia-Ova
Listeria-Ova
Undivided
1 Division
2 Divisions
3 Divisions
4 Divisions
5 Divisions
6 Divisions
7 Divisions
***
**
*

Figure 5-1: *Listeria Monocytogenes* Vaccinations Drive CD8$^+$ T Cell Divisions Without Up Regulation of the Checkpoint Proteins PD-1 and LAG-3

CFSE labeled OT-1s were admixed with their vaccine and then adoptively transferred into recipient host mice, either congenically marked CD45.1$^+$ wildtype or CAG-OVA mice. On day three, spleens were harvested, and splenocytes stained intracellularly for transcription factor expression. A) Cells were gated on CFSE expression as well as CD8 and CD45.2, before being examined as for expression of individual checkpoint proteins and B) quantified. Data shown are representative of greater than 3 independent experiments, with n=3-4 mice per group.

Figure 5-2


A)
C57/B6
Cag-Ova
Vaccinia-Ova
Listeria-Ova
WT
PD-1 KO
PD-1
0.0
0.2
0.7
99.0
86.1
0.0
13.8
0.0
40.7
0.0
59.1
0.2
6.3
0.0
0.0
0.8
99.3
0.5
0.0
99.0
0.5
0.2
0.0
99.7
0.1
0.6
0.0
99.2
0.2
CFSE

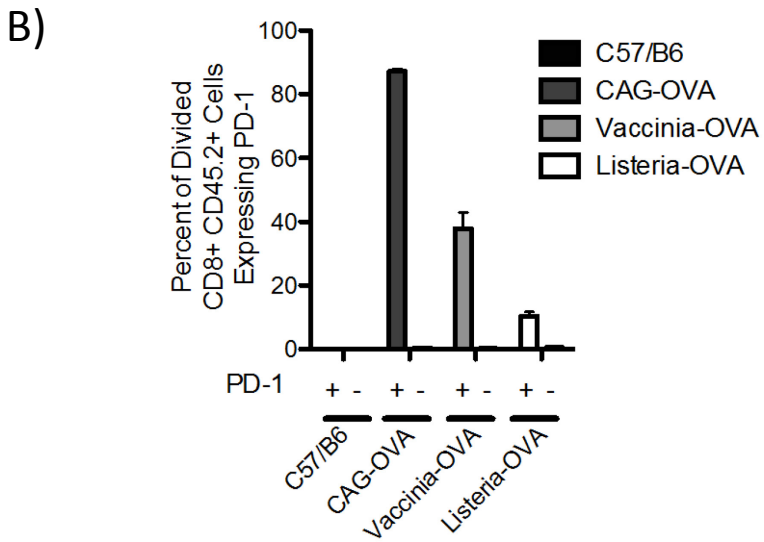
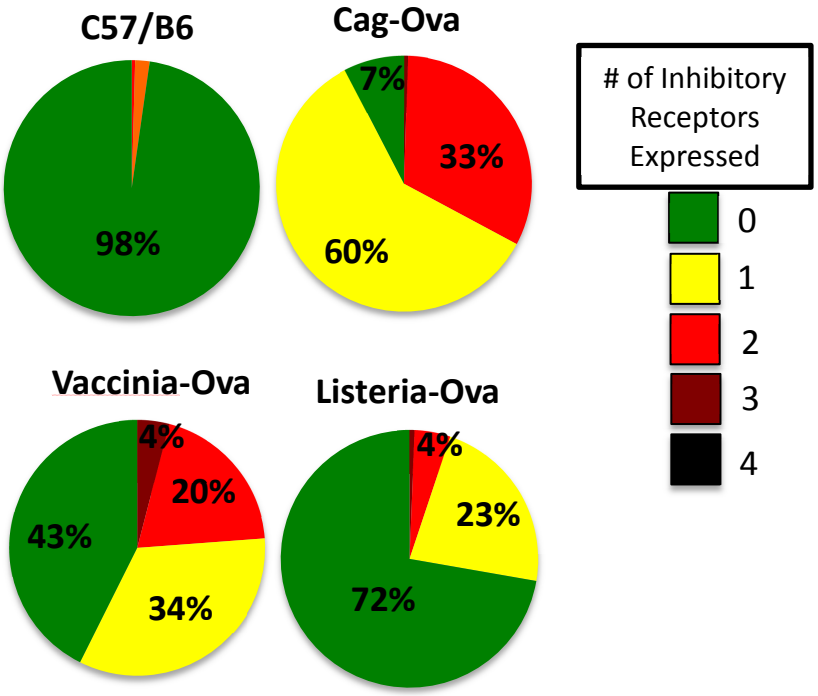

B)
Percent of Divided CD8+ CD45.2+ Cells Expressing PD-1
100
80
60
40
20
0
C57/B6
CAG-OVA
Vaccinia-OVA
Listeria-OVA
PD-1
+ -
+ -
+ -
+ -
C57/B6
CAG-OVA
Vaccinia-OVA
Listeria-OVA

Figure 5-2: *Listeria Monocytogenes* Vaccinations Result in CD8$^+$ T Cell Activation Without PD-1 Expression

CFSE labeled wildtype or PD-1$^{-/-}$ OT-1s were admixed with their vaccine and then adoptively transferred into recipient host mice, either congenically marked CD45.1$^+$ wildtype or CAG-OVA mice. On day three, spleens were harvested, and splenocytes stained for PD-1 expression. A) Dot plots of PD-1 expression on donor OT-I CD8$^+$ T cells and B) quantification of PD-1 expression. Data shown are representative of at least 2 independent experiments, with n=3-4 mice per group.

Figure 5-3

A)


C57/B6
98%
Cag-Ova
7%
33%
60%
# of Inhibitory Receptors Expressed
0
1
2
3
4
Vaccinia-Ova
4%
20%
43%
34%
Listeria-Ova
4%
23%
72%

B)

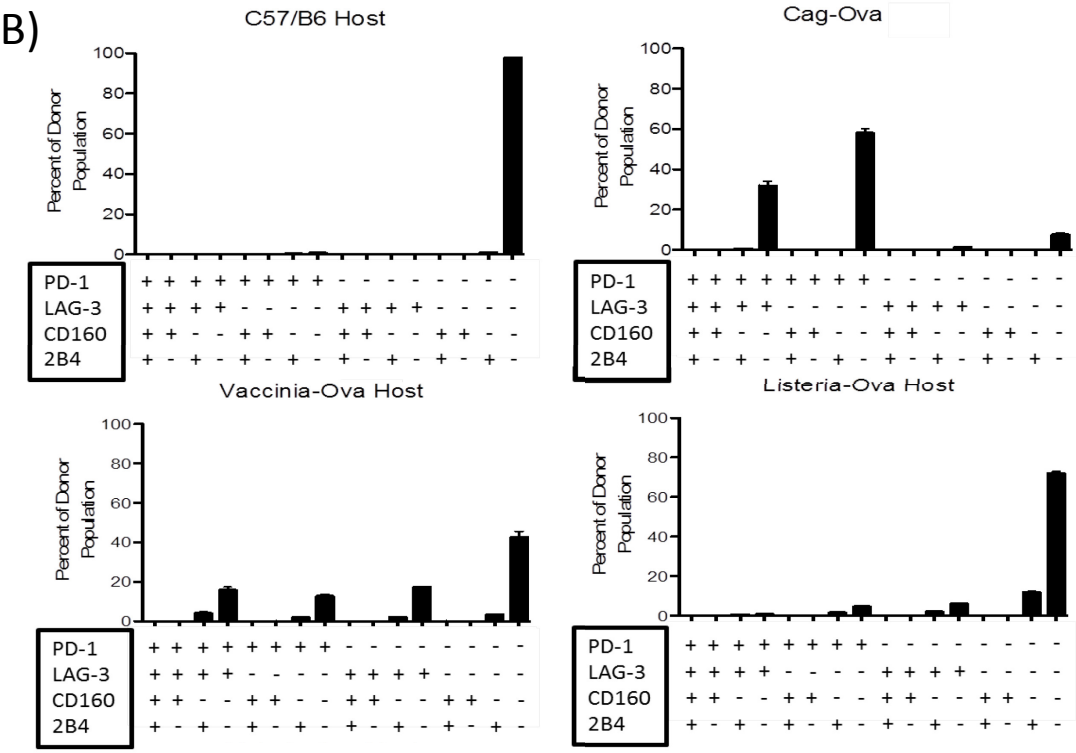

C57/B6 Host
Cag-Ova
Vaccinia-Ova Host
Listeria-Ova Host
Percent of Donor Population
PD-1
LAG-3
CD160
2B4

Figure 5-3: *Listeria Monocytogenes* Vaccinations Drive CD8$^+$ T Cells To Divide Without Combinatorial Expression of Checkpoint Proteins

CFSE labeled OT-1s were admixed with their vaccine and then adoptively transferred into the same host mice as previous experiments. On day three, spleens were harvested, and splenocytes stained for checkpoint protein expression. Cells were gated on CFSE expression as well as CD8 and CD45.2, before being examined as for expression of A) multiple checkpoint proteins or B) patterns of checkpoint proteins. Data shown are representative of greater than 3 independent experiments, with n=3-4 mice per group.

Figure 5-4

A)

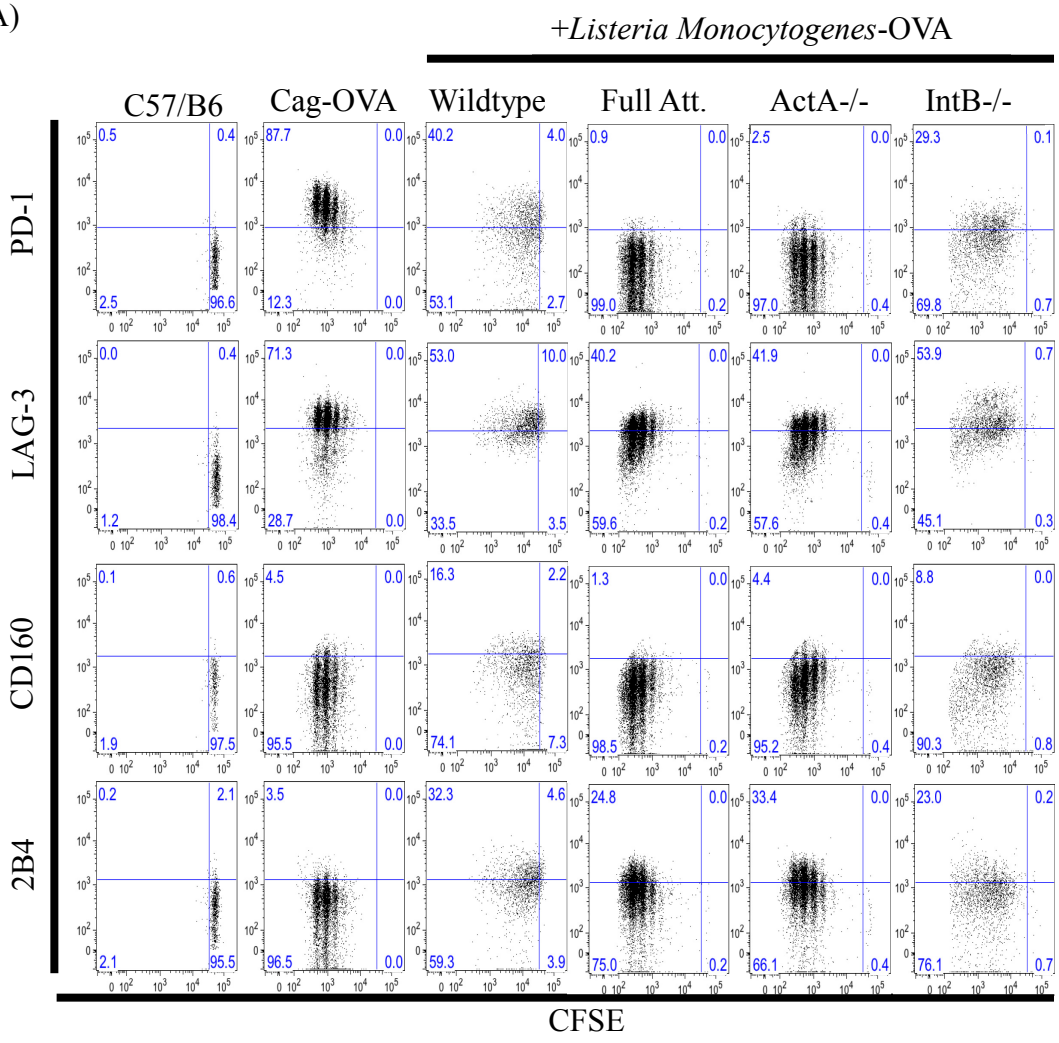

+Listeria Monocytogenes-OVA
C57/B6
Cag-OVA
Wildtype
Full Att.
ActA-/-
IntB-/-
PD-1
LAG-3
CD160
2B4
CFSE

Figure 5-4: Activation of $CD8^+$ T Cells Without PD-1 Expression is Dependent on the ActA Deletion in the Live, Attenuated *Listeria Monocytogenes* Vector

CFSE labeled OT-1s were admixed with live-attenuated strains of *Listeria Monocytogenes* with different genetic attenuations, before being adoptively transferred into recipient host mice, either congenically marked $CD45.1^+$ wildtype or CAG-OVA mice. On day three, spleens were harvested, and splenocytes stained for checkpoint protein expression. Cells were gated on CFSE expression as well as CD8 and CD45.2, before being examined as for expression of individual checkpoint proteins. Data shown are representative of at least 2 independent experiments, with n=3 mice per group.

Figure 5-5

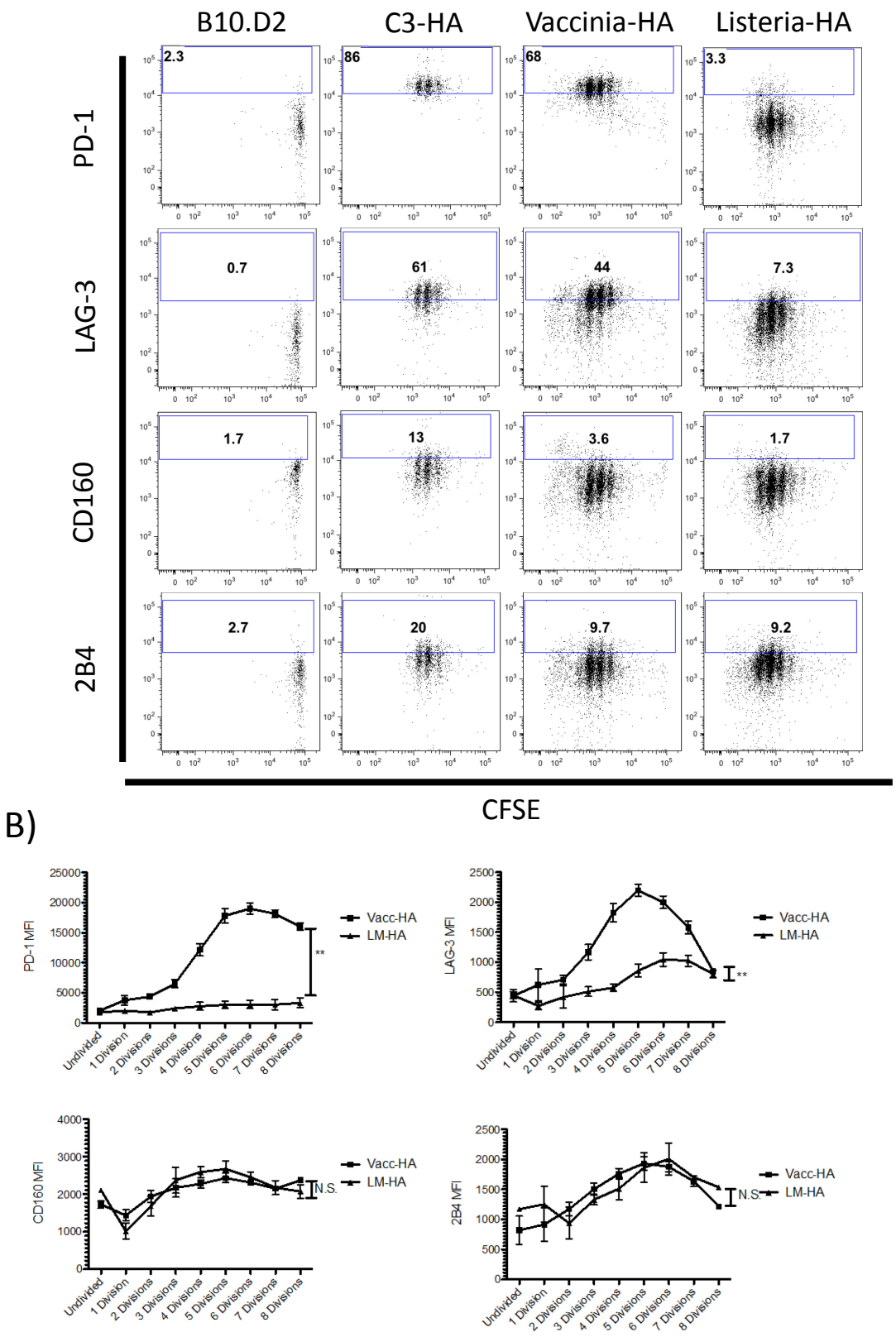

A)
B10.D2
C3-HA
Vaccinia-HA
Listeria-HA
PD-1
LAG-3
CD160
2B4
2.3
86
68
3.3
0.7
61
44
7.3
1.7
13
3.6
1.7
2.7
20
9.7
9.2
CFSE
B)
PD-1 MFI
LAG-3 MFI
CD160 MFI
2B4 MFI
Vacc-HA
LM-HA
**
N.S.
Undivided
1 Division
2 Divisions
3 Divisions
4 Divisions
5 Divisions
6 Divisions
7 Divisions
8 Divisions

Figure 5-5: Activation Without Expression of Checkpoint Proteins Is Not A Function of the OT-I Adoptive Transfer Model

CFSE labeled Clone 4s were admixed with their vaccine and then adoptively transferred into recipient host mice, either congenically marked Thy1.2^{+} wildtype or C3-HA mice. On day three, spleens were harvested, and splenocytes stained intracellularly for transcription factor expression. A) Cells were gated on CFSE expression as well as CD8 and Thy1.1, before being examined as for expression of individual checkpoint proteins and B) MFI of individual checkpoints as a function of division. Data shown are representative of greater than 3 independent experiments, with n=4 mice per group.

Figure 5-6

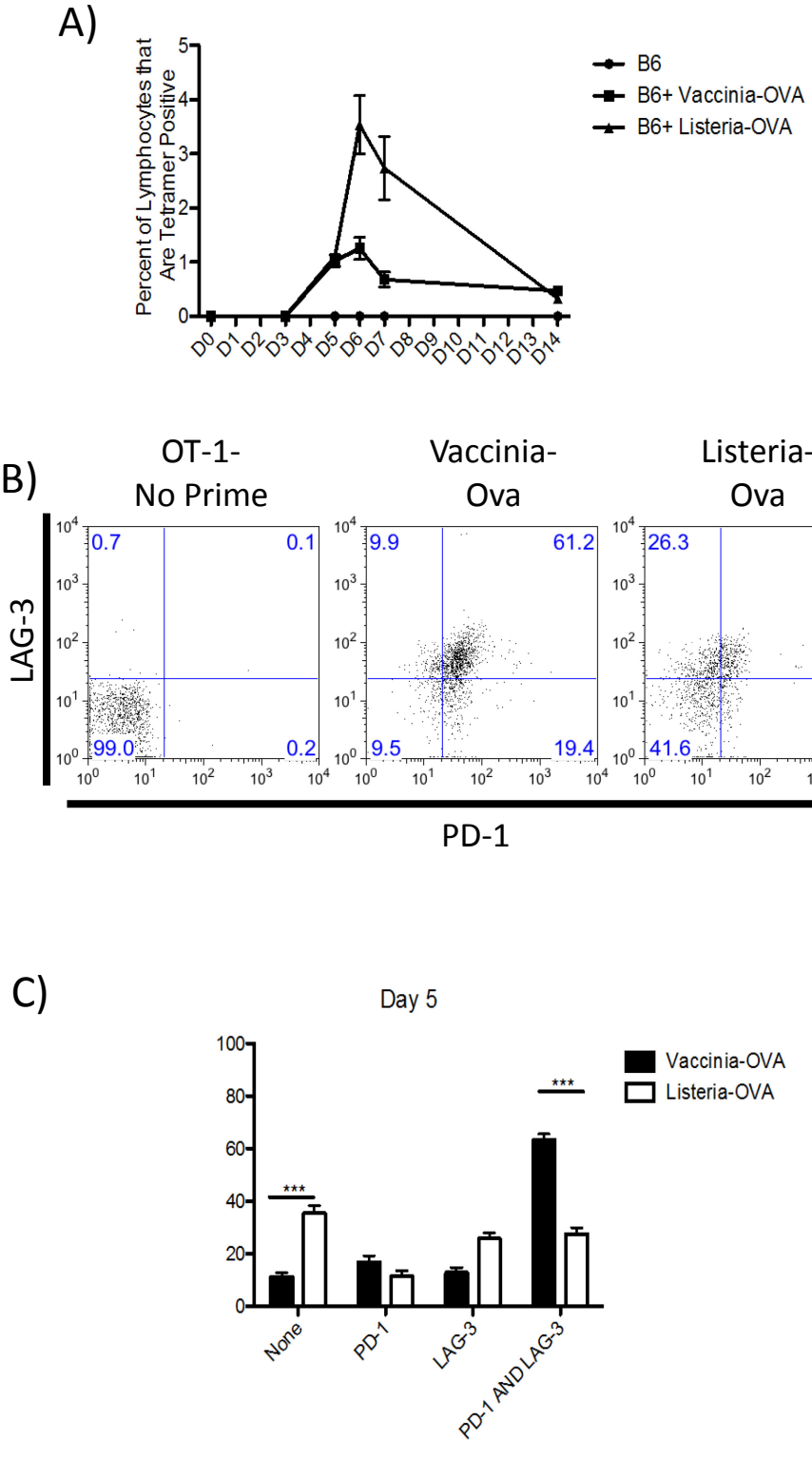

A)
Percent of Lymphocytes that Are Tetramer Positive
B6
B6+ Vaccinia-OVA
B6+ Listeria-OVA
D0 D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14
B)
OT-1- No Prime
Vaccinia- Ova
Listeria- Ova
LAG-3
PD-1
0.7
0.1
99.0
0.2
9.9
61.2
9.5
19.4
26.3
22.3
41.6
9.9
C)
Day 5
Vaccinia-OVA
Listeria-OVA
***
***
None
PD-1
LAG-3
PD-1 AND LAG-3

Figure 5-6: Endogenous Antigen Specific $CD8^+$ T cells Generated in Response to *Listeria* Vaccination Have Significantly Attenuated PD-1 and LAG-3 Co-Expression

C57/B6 mice were vaccinated on Day 0 with either PBS, 10^6 pfu Vaccinia-OVA, or 10^7 cfu *Listeria*-OVA, and peripheral blood was collected daily and stained for A) OVA tetramer specificity, as well as B) PD-1 and LAG-3 expression on Day 5. One C57/B6 OT-1 mouse was bled daily as a positive control for tetramer staining. C) Quantification of checkpoint co-expression. Data shown are representative of at least 2 independent experiments, with n=4 mice per group.

Figure 5-7

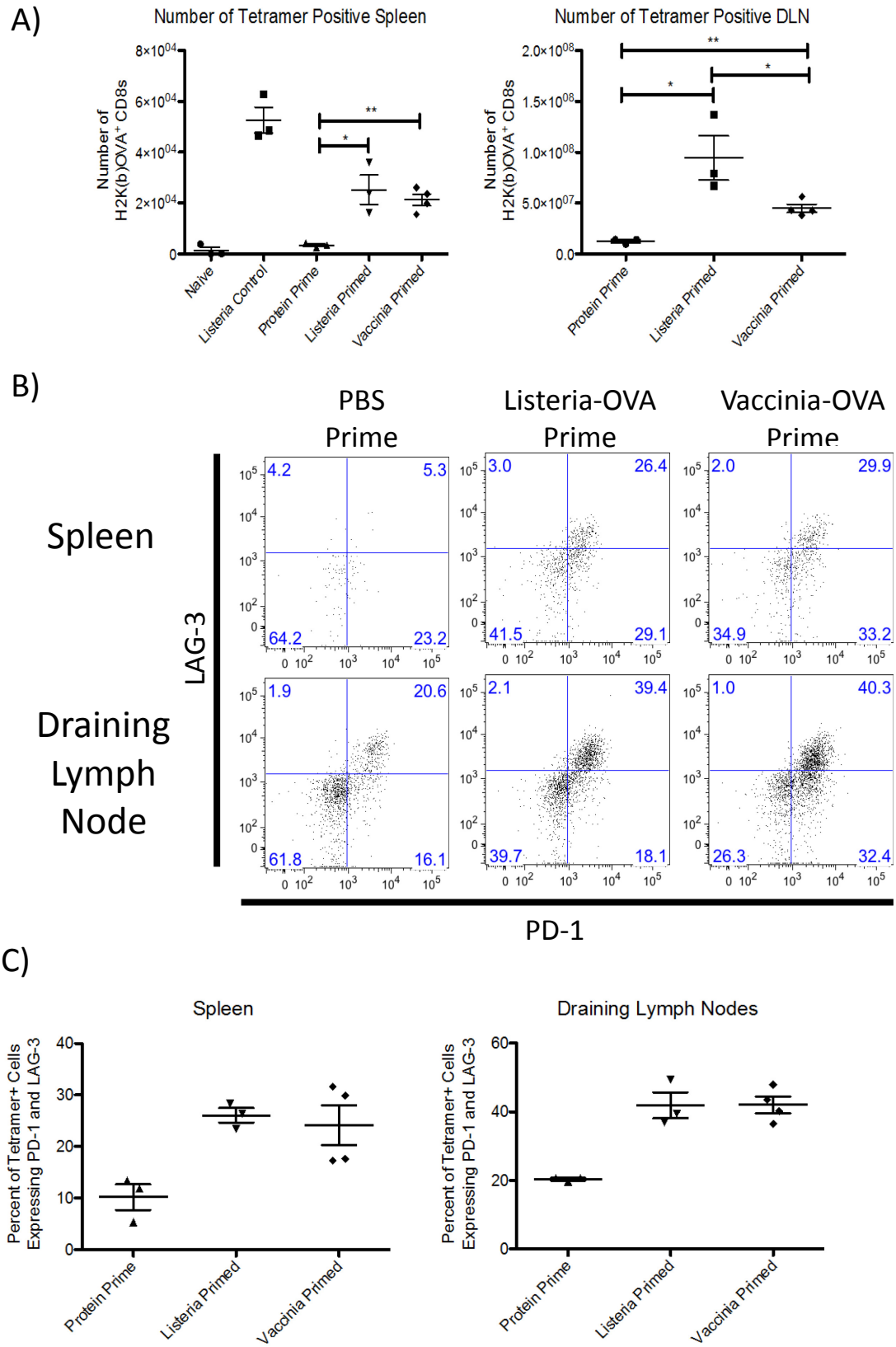

A)
Number of Tetramer Positive Spleen
Number of H2K(b)OVA+ CD8s
Naive
Listeria Control
Protein Prime
Listeria Primed
Vaccinia Primed
Number of Tetramer Positive DLN
Protein Prime
Listeria Primed
Vaccinia Primed
B)
PBS Prime
Listeria-OVA Prime
Vaccinia-OVA Prime
Spleen
Draining Lymph Node
LAG-3
PD-1
4.2
5.3
64.2
23.2
3.0
26.4
41.5
29.1
2.0
29.9
34.9
33.2
1.9
20.6
61.8
16.1
2.1
39.4
39.7
18.1
1.0
40.3
26.3
32.4
C)
Spleen
Draining Lymph Nodes
Percent of Tetramer+ Cells Expressing PD-1 and LAG-3
Protein Prime
Listeria Primed
Vaccinia Primed

Figure 5-7: *Listeria* Vaccinations Do Not Control Future PD-1 Expression During Recall Responses

C57/B6 mice were primed with either PBS, Vaccinia-OVA, or *Listeria*-OVA. On Day 40, mice were rechallenged subcutaneously with whole OVA protein mixed with Addavax adjuvant, and spleens and draining lymph nodes were harvested 5 days later and stained for A) tetramer positivity, as well as B-C) PD-1 and LAG-3 expression. Unvaccinated mice were used as a negative control for tetramer staining, while some mice receiving LM-OVA on day 40 as a positive control for tetramer staining. Data shown are representative of at least 2 independent experiments, with n=3 mice per group.

Figure 5-8

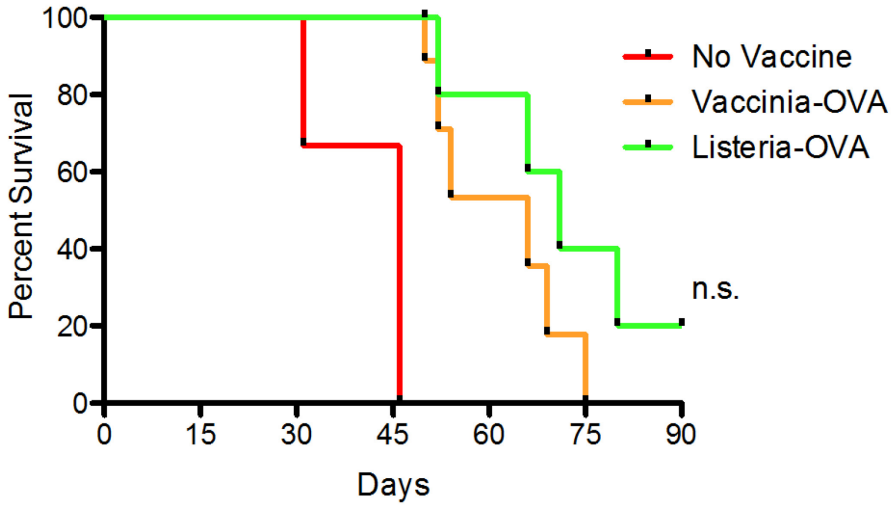


Figure 5-8: PD-1 Expression During Vaccination is One of the Major Molecular Drivers of Differences in Functional Anti-Tumor Immunity

PD-1$^{-/-}$ host mice were injected intravenously with 1-1.5x10^{5} B16-OVA. Three days later, mice were vaccinated with either PBS, *Listeria*-OVA, Vaccinia-OVA via intravenous injection and monitored for survival over time. Data shown are representative of at least 2 independent experiments, with n=3-5 mice per group. Statistics compare the overall survival of the Vaccinia-OVA vaccination group vs. the Listeria-OVA vaccination group

CHAPTER VI

$CD8^+$ T CELL ACTIVATION WITHOUT PD-1 EXPRESSION IS INDEPENDENT OF MYD88 OR STING PATHWAYS IN ANTIGEN PRESENTING CELLS

Introduction

Several groups have begun to characterize the different pathways stimulated by *Listeria Monocytogenes*. In these studies, it has been found that heat killed *Listeria Monocytogenes* based vectors stimulate antigen presenting cells differently than metabolically active *Listeria*[130,159]. Indeed, *Listeria* infections have been reported to be sensed both by extracellular[121] as well as intracellular[122–125] pathogen associated pattern receptors. However, the final result of many of these sensors, and the only known pathway required for the production of IFNβ, involves TBK1 and IRF3[107]. Yet, *in vivo*, there is likely some overlap between the initial set of sensors registering the infection and the final signaling pathway. MyD88 is a crucial adaptor protein for many of the innate pattern recognition receptors, including many of the Toll-like receptors[160]. Studies have shown both MyD88 dependent[121] and independent mechanisms of responding to *Listeria Monocytogenes* infection. Additional recent work has identified the potential for the STING and RIGI pathways in the recognition of *Listeria* by host cells[122–124]. In this chapter, we examined many of the hallmarks of *Listeria* infection that we thought may be capable of the unique $CD8^+$ T cell activation phenotype that is seen when using this live-attenuated strain of *Listeria Monocytogenes.*

Chapter Specific Materials and Methods

In Vitro Stimulation With IFNβ

CD45.1+ C57/B6 wildtype splenocytes were depleted of $CD19^+$ B cells and $CD3\varepsilon^+$ T cells using magnetic bead enrichment, following manufacture's protocol (Miltenyi, San Diego CA). Following depletion, different concentrations of OVA I

peptide were added to the depleted presenting cells, such that the final concentrations would be 1pM, 1nM, and 1 μM once plated *in vitro.* Naïve, CD8 enriched, CFSE labeled OT-1 cells were prepared as previously described, and $1x10^5$ OT-1s were put into culture with $4x10^5$ presenters along with 2ng/mL mIL-2. Each condition was plated in triplicate, +/- 100 units of recombinant human IFNβ (Peprotech, Rocky Hill, NJ) for 72 hours before checkpoint expression was investigated using flow cytometry as previously described

Direct Ex Vivo Antigen Detection Assay With Experienced T Cell Responders

To lower the threshold for IFNγ production, this assay was first performed with previously activated responding OT-1 T cells. Briefly, whole splenocytes from a OT-1 $Rag2^{-/-}$ mouse were pulsed with 1 nM OVA I peptide, and 2ng/mL murine IL-2 (Peprotech, Rocky Hill, NJ). Cells were spun down and resuspended in new media, containing 2ng/mL IL-2 on days 2 and 4. On Day 5, $CD8^+$ T cells were isolated by magnetic bead isolation (Miltenyi, San Diego, CA), and CFSE labeled with 5μM as previously described. In parallel, mice were infected on a staggered timeline with 10^6 pfu Vaccinia-OVA or 10^7 cfu *Listeria*-OVA, such that, when spleens were harvested on day 5, vaccinations had been *in vivo* for 72, 48, 24, and 12 hours. From these presenters, spleens were harvested as previously described, RBCs were lysed, and $2x10^5$ presenting cells were plated with $2x10^4$ CFSE labeled, antigen experienced OT-1s for 6 hours. Following the first six hour incubation, protein transport inhibitor cocktail was added, and an additional six hour culture was performed, before cells were stained for IFNγ

expression as a readout of OVA presentation. For controls, known concentrations of peptide were incubated with T cell depleted splenocytes as presenters.

Cyclic Dinucleotide Stimulation

$CD8^+$ OT-1 T cells were enriched as previously described. 1-1.5x10^6 cells were adoptively transferred into congenically marked host mice ($CD45.1^+$). One day alter, mice were vaccinated subcutaneously with either Addavax emulsion (Invivogen, San Diego, CA) with 10μg of Endofit whole OVA protein (Invivogen, San Diego, CA), +/- 76μg of cyclic dinucleotides (Aduro Biotech, Berkley, CA). As a control for activation without PD-1 up regulation, some mice were vaccinated intraperitoneally with 10^7 *Listeria*-OVA. On day 6 following vaccination, draining lymph nodes were harvested, and stained to identify adoptively transferred cells as well as expression of checkpoint proteins in response to the two vaccines.

Results

Production of IFNβ By Host Cells Is Not Responsible for $CD8^+$ T cell Activation Without PD-1 Expression

IFNβ is a type I interferon protein, and as such, is often a characteristic of responses to viral pathogens. However, several groups have identified IFNβ production as a key feature of innate immune system's response to *Listeria Monocytogenes* [130]. To test if exposure of $CD8^+$ T cells to IFNβ was responsible for CD8+ T cell activation without expression of PD-1, we designed an *in vitro* model of $CD8^+$ T cell activation where we titrated in increased amounts of OVA peptide to simulate different levels of

TCR stimulation in the presence or absence of IFNβ. Despite its role in generation of the innate immune response, and its ability to up regulate CD69 on for $CD8^+$ T cells (Fig 6-1a), IFNβ signaling did not decrease expression of PD-1, and in fact, may have increased expression of both PD-1 and LAG-3, and subsequently decreased overall divisions (Fig 6-1b,c). Therefore, skewing of the $CD8^+$ T cells by the presence of excess IFNβ during the generation of the immune response was not responsible for the activation of $CD8^+$ T cells without PD-1 expression

$CD8^+$ T Cell Activation Without PD-1 Expression Is Not A Function of Decreased Peptide Presentation By Listeria-OVA Vaccination

However, when $CD8^+$ T cells were stimulated with a low dose of OVA peptide, in the absence of IFNβ we did see activation of the OT-1s without up regulation of PD-1 (Fig 6-1b,c), suggesting to us that perhaps, these two vaccinations result in different amounts of OVA peptide being presented in the context of MHC I. Therefore, to test if *Listeria* vaccines present less OVA peptide than Vaccinia vaccines, we utilized a Direct *Ex vivo* Antigen Detection (DEAD) Assay, where we co-cultured experienced OT-1s with splenocytes from vaccinated mice at different time points. Since experienced $CD8^+$ T cells are licensed and primed, they are capable of detecting extremely low levels of their target peptide, and responding through the production of IFNγ along with other cytolytic effectors molecules. As a control, we also co-cultured our experienced OT-1s with splenocytes pulsed with different concentrations of OVA peptide. Surprisingly, we found that these vaccines present OVA peptide with different kinetics. Vaccinia-OVA vaccination results in early peak display of MHC I:OVA complex, while *Listeria*-OVA

requires 24 hours to reach the peak of MHC:OVA expression (Fig 6-2a,b). Furthermore, *Listeria* based vaccination resulted in significantly more MHC:OVA expression, though both vaccines were in the range of 1-5pM effective peptide presentation when compared with peptide pulsed controls. Therefore, lack of PD-1 expression on $CD8^+$ T cells primed against attenuated *Listeria Monocytogenes* was not due to decreased TCR signaling, as *Listeria* based vaccinations actually presented more peptide to experienced OT-1s.

Activation of $CD8^+$ T Cells Without PD-1 Up Regulation Is Independent of MyD88 Expression By Host Antigen Presenting Cells

MyD88 is an adaptor protein that has been found to be important in the identification and integration of innate immune signals within the immune system, including the development and maturation of dendritic cells. Indeed, MyD88 knockout animals have increased susceptibility to a variety of bacterial pathogens, since all Toll-Like Receptors (except TLR3) signal through MyD88 recruitment[160]. To examine whether $CD8^+$ T cell activation without PD-1 up regulation was a function of one of the MyD88 dependent toll-like receptor signaling pathways, we utilized MyD88 knockout host animals. After vaccination with *Listeria*-OVA, we examined the expression of checkpoint proteins on adoptively transferred OT-1s in wildtype and MyD88 knockout hosts. Interestingly, despite the large number of innate immune receptors that signal through MyD88, MyD88 knockout host animals were equally capable of generating $CD8^+$ T cell activation without PD-1 up regulation (Fig 6-3a,b). However, we did see significant up regulation of LAG-3 on the responding $CD8^+$ T cells. While the molecular mechanisms that govern the expression of individual checkpoint proteins are not completely understood, these data suggest that there are individual pathways in the

antigen presenting cells responsible for modulating the expression of individual checkpoint proteins on the responding $CD8^+$ T cell population, and that checkpoint co-expression is not completely redundant. These data further suggest that the molecular signaling pathway responsible for generating $CD8^+$ T cell activation without PD-1 expression by attenuated *Listeria Monocytogenes* is independent of MyD88, and therefore independent of all MyD88 signaling pathways, including many of the TLRs.

Stimulation of the STING Pathway is Not Sufficient To Generate $CD8^+$ T cell Activation Without PD-1 Up Regulation

Another pathway that has recently been implicated in the host sensing of *Listeria Monocytogenes* is the Stimulator of Interferon Genes (STING) pathway. STING is an intracellular signaling receptor that has been reported to sense the cyclic dinucleotides produced in large quantities by *Listeria*, as well as several other pathogens[124]. To test the role STING plays in the priming of $CD8^+$ T cells to *Listeria* based vaccines, we attempted to intentionally stimulate the STING pathways during $CD8^+$ T cell activation. Utilizing Addavax as the emulsion, we vaccinated mice with subcutaneous whole OVA protein with or without the addition of cyclic dinucleotides, the ligands for STING. In this model, we found that stimulating the STING pathway was not sufficient to generate a CD8+ T cell phenocopy of vaccination with *Listeria*-OVA, and that the addition of CDNs may have actually increased checkpoint expression rather than decrease it (Fig6-4a,b). These data suggest that it is not simply stimulation of the STING pathway that prevents PD-1 up regulation, though it does not discount the requirement for STING in combination with other pathways.

STING Pathway Signaling is Not a Requirement For The Generation Of Anti-Tumor Immunity By Live, Attenuated Listeria Vaccination

We also wanted to examine the effects of STING signaling in the generation of anti-tumor immunity by either Vaccinia or *Listeria* vaccinations. To test this, we gave STING knockout animals B16-OVA intravenously, followed by the same vaccination strategy as previously described. In agreement with the CDN stimulation studies, we found *Listeria* vaccinations still had significantly decreased tumor burden (Fig 6-5a,b) as well as superior overall survival (Fig 6-5c), suggesting that STING is not required for the generation of anti-tumor immunity to *Listeria* based vaccinations.

Summary

The generation of a successful $CD8^+$ T cell response is dependent on the recognition of the pathogen by the innate immune system, specifically by professional antigen presenting cells, such as dendritic cells. Following antigen recognition, dendritic cells mature and present antigens to $CD8^+$ and $CD4^+$ T cells to prime subsequent expansion and maturation of these cells. The pathways that are stimulated by an individual pathogen depend on characteristics of that pathogen, including the production of 5' triphosphorylated RNA, flagellin expression, or a myriad of other pathogen associated molecular patterns. In the case of *Listeria*, many different pathways all seem to play some role in the maturation of the innate immune response, as well as the generation of a potent adaptive immune response. However, IFNβ production, MyD88 signaling, and STING signaling were all unnecessary for the generation of a CD8+ T cell activation phenotype with PD-1 expression. These data support one of two hypothesis.

The first is that these pathways overlap, and therefore combinations of their signaling results in the CD8+ T cell activation phenotype we observe when using this live-attenuated strain of *Listeria*. A second hypothesis is that stimulation of a specific pathway, not examined here, is responsible for the $CD8^+$ T cell phenotype. One pathway we did not examine here was RIG-I stimulation, as the *in vivo* tools for examining this pathway are only recently becoming available. However, together these data demonstrate that STING and MyD88 are likely dispensable for activation of $CD8^+$ T cells without up regulation of PD-1.

Figure 6-1

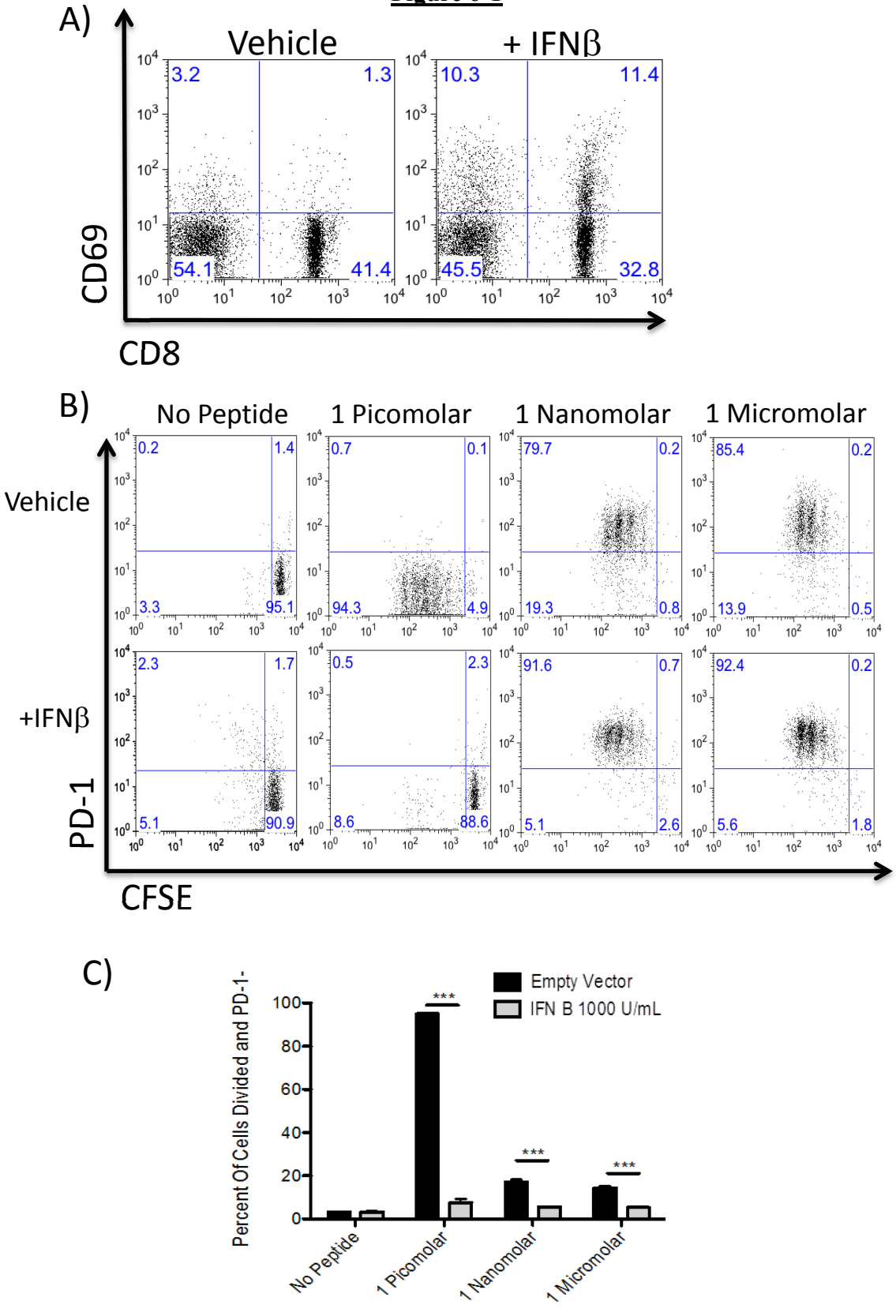

A)
Vehicle
+ IFNβ
3.2
1.3
54.1
41.4
10.3
11.4
45.5
32.8
CD69
CD8
B)
No Peptide
1 Picomolar
1 Nanomolar
1 Micromolar
Vehicle
+IFNβ
0.2
1.4
3.3
95.1
0.7
0.1
94.3
4.9
79.7
0.2
19.3
0.8
85.4
0.2
13.9
0.5
2.3
1.7
5.1
90.9
0.5
2.3
8.6
88.6
91.6
0.7
5.1
2.6
92.4
0.2
5.6
1.8
PD-1
CFSE
C)
Empty Vector
IFN B 1000 U/mL
Percent Of Cells Divided and PD-1-
100
80
60
40
20
0
***
***
***
No Peptide
1 Picomolar
1 Nanomolar
1 Micromolar

Figure 6-1: Activation of $CD8^+$ T Cells Without PD-1 Expression is Not Due To IFNβ Skewing by Host Cells

A) Splenocytes were incubated overnight at in 100U/mL of rIFNβ before being stained for CD69 up regulation on $CD8^+$ T cells. B) CFSE labeled OT-1s were cultured with splenocytes pulsed with different concentrations of OVA peptide +/- 100U/mL of rIFNβ for 3 days before being stained for PD-1 expression. C) Repeat wells were quantified for PD-1 expression. Data shown are representative of at least 2 independent experiments, with triplicate wells plated for each condition.

Figure 6-2

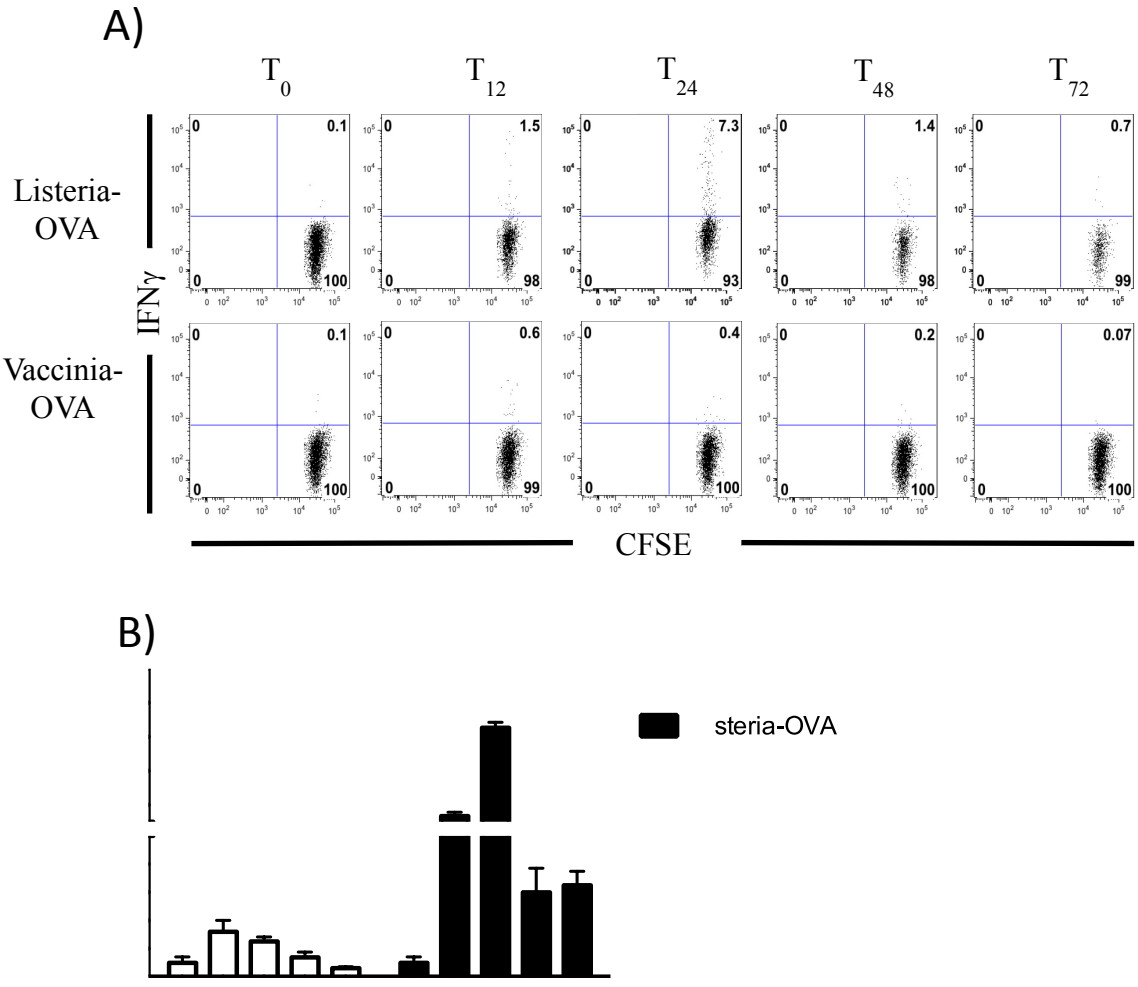

A)
T0
T12
T24
T48
T72
Listeria-OVA
Vaccinia-OVA
IFNγ
CFSE
0.1
1.5
7.3
1.4
0.7
100
98
93
98
99
0.1
0.6
0.4
0.2
0.07
100
99
100
100
100
B)
steria-OVA

Figure 6-2: *Listeria* Vaccination Produces Increased Functional Presentation of OVA Peptide

Host mice were given staggered vaccinations of either Vaccinia-OVA or *Listeria*-OVA, and harvested after the indicated time *in vivo*. $2x10^5$ splenocytes were co-cultured with $2x10^4$ CFSE labeled, previously activated, OT-I $CD8^+$ T cells for 6 hours in the presence of protein transport blockade. Following co-culture, cells were directly stained for IFNγ production. A) Dot plots gated on $CFSE^+$ $CD8^+$ T cells. B) Quantification of replicate wells. Data shown are representative of at least 2 independent experiments, with triplicate wells plated for each condition.

Figure 6-3

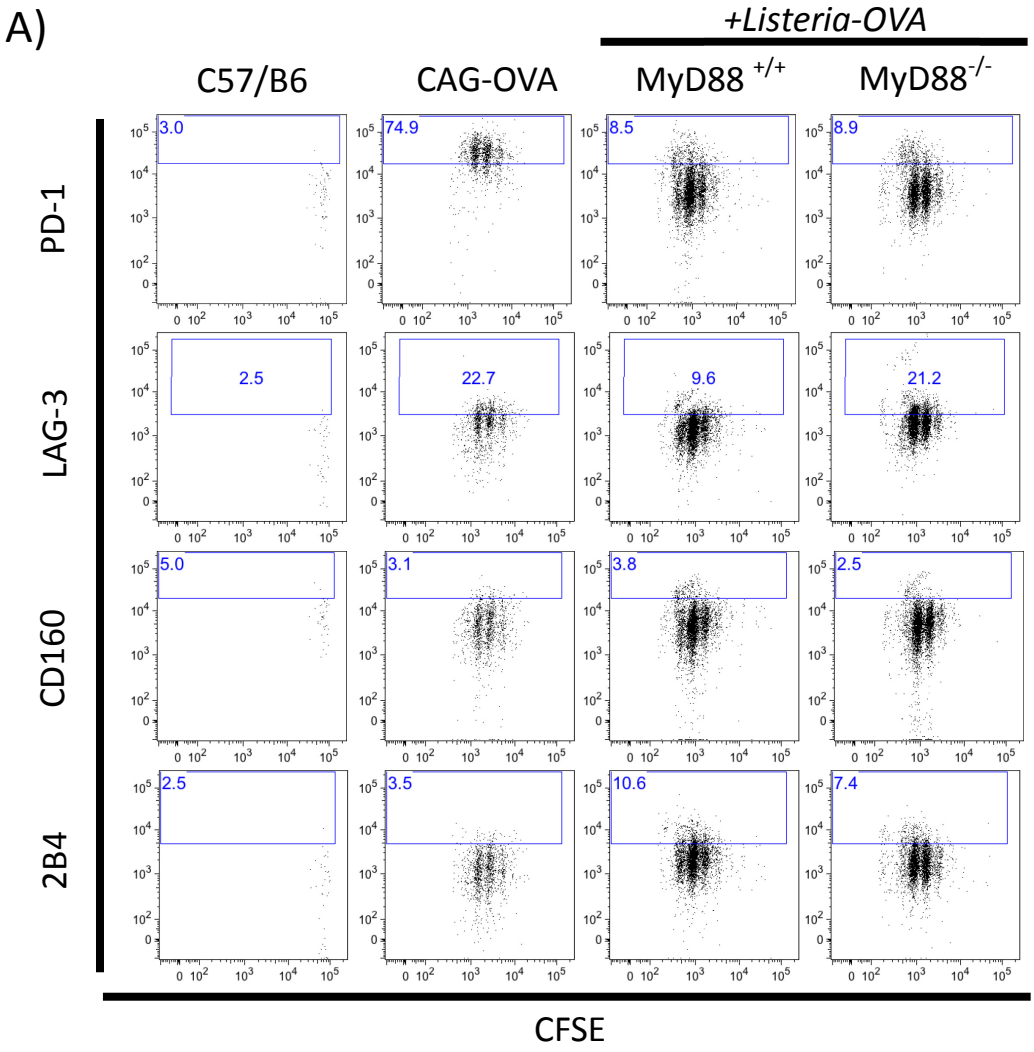

A)
+Listeria-OVA
C57/B6
CAG-OVA
MyD88 +/+
MyD88 -/-
PD-1
LAG-3
CD160
2B4
3.0
74.9
8.5
8.9
2.5
22.7
9.6
21.2
5.0
3.1
3.8
2.5
2.5
3.5
10.6
7.4
CFSE
B)
Percent PD-1 Positive
N.S.
Percent LAG-3 Positive
**
Percent CD160 Positive
N.S.
Percent 2B4 Positive
*
C57/B6 Host
Cag-Ova
WT
MyD88 KO

Figure 6-3: *Listeria* Vaccine Driven CD8^{+} T Cell Activation Without PD-1 Up Regulation Is Mediated By A Pathway Independent of MyD88

CFSE labeled OT-1s were admixed with their vaccine and then adoptively transferred into recipient host mice, either congenically marked CD45.1^{+} wildtype or MyD88 $^{-/-}$ mice, or CAG-OVA mice for a tolerizing control. On day three, spleens were harvested, and splenocytes stained for checkpoint protein expression. A) Cells were gated on CFSE expression as well as CD8 and CD45.2, before being examined as for expression of individual checkpoint proteins and B) quantified. Data shown are representative of at least 2 independent experiments, with n=4 mice per group.

Figure 6-4

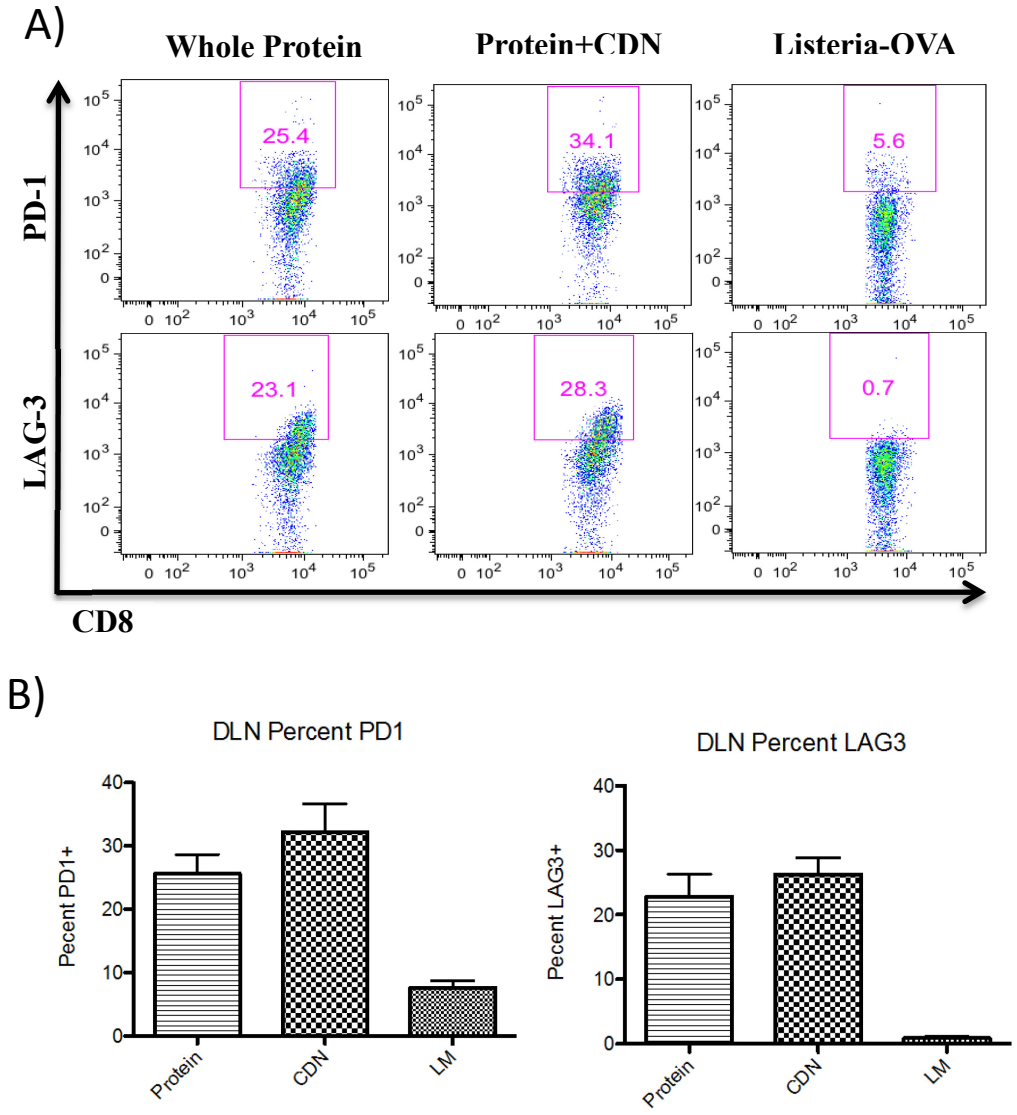

A)
Whole Protein
Protein+CDN
Listeria-OVA
PD-1
LAG-3
CD8
25.4
34.1
5.6
23.1
28.3
0.7
B)
DLN Percent PD1
Pecent PD1+
Protein
CDN
LM
DLN Percent LAG3
Pecent LAG3+
Protein
CDN
LM

Figure 6-4: Stimulation of the STING Pathway is Not Sufficient for $CD8^+$ T Cell Activation Without PD-1 Up Regulation

OT-1 T cells were magnetically enriched as previously described, and transferred into new hosts. One day later, host mice were challenged subcutaneously with Addavax + OVA protein, with or without the addition of cyclic dinucleotides. Some mice were challenged with *Listeria*-OVA as a control for activation without PD-1 up regulation. 5 days following challenge, adoptively transferred cells were examined in the challenge draining lymph nodes. A) Dot plots of PD-1 and LAG-3 expression and B) Quantification of checkpoint protein expression. Data shown represent at least 2 independent experiments, with n=3 mice per group.

Figure 6-5

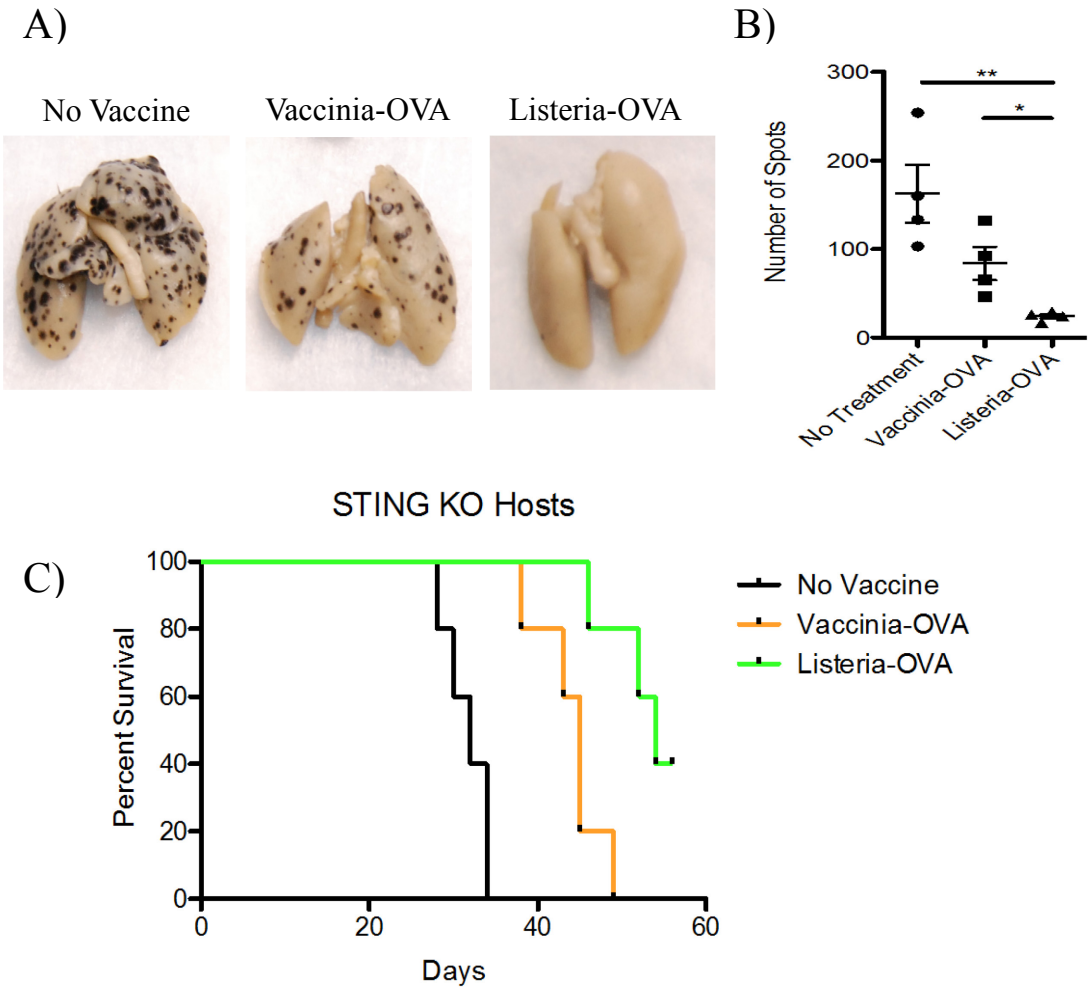


Figure 6-5: The Protective Immunity Generated by *Listeria Monocytogenes* Vaccination is Independent of STING Signaling

STING$^{-/-}$ host mice were injected intravenously with 1-1.5x10^5 B16-OVA. Three days later, mice were vaccinated with either PBS, 10^7 cfu of *Listeria*-OVA, or 10^6 pfu of Vaccinia-OVA via intravenous injection. Mice were then A-B) harvested on day 18 for measurement of tumor load or C) monitored for survival. Data shown represent at least 2 independent experiments, with n=4 mice per group.

CHAPTER VII

LISTERIA MONOCYTOGENES VACCINATIONS INDUCE A UNIQUE TRANSCRIPTIONAL PROGRAM IN CD8α$^+$ DENDRITIC CELLS

Introduction

Professional antigen presenting cells, such as dendritic cells, are responsible for the generation of potent adaptive immune responses[161,162]. However, it is now understood that the primary populations of professional antigen presenting cells are not one homogenous population, but instead made up of many different subsets based on function and location. Dendritic cells, originally identified by Paul Langerhans, represent the major functional population of cells capable of priming adaptive immunity through the activation and subsequent licensing of $CD4^+$ and $CD8^+$ T cells to generate a primary response, as well as develop a memory response for lifelong protective immunity. Recent work by a variety of labs has demonstrated that spleen and lymph node resident dendritic cells can also be broadly divided into several subgroups. One of these groups, the $CD8\alpha^+$ dendritic cell subset, has recently been implicated in the presentation of tumor associated antigens to the adaptive immune system[163].

$CD8\alpha^+$ dendritic cells ($CD8\alpha^+$ DC) require the transcription factor BATF3[164] as well as FLT-3 signaling[165,166], and have been found to have highly efficient cross presentation potential[167–169]. This means they can efficiently transfer antigens found outside the cell onto an MHC I protein, which is normally restricted to internal antigens, allowing these cells to generate $CD8^+$ T cell responses to external antigens. Furthermore, the cross presentation potential of this population is likely linked to its capacity to scavenge dead and dying cells[168], and is crucially important in the generation of anti-tumor $CD8^+$ T cell responses. Furthermore, prior to activation, this subset of dendritic cells plays a role in tolerizing the immune response to dying cells[170]. Interestingly, $CD8\alpha^+$ DCs may require type I interferons to fully mature and promote $CD8^+$ T cell

responses[171], suggesting a role for the interplay of these different dendritic cell subsets. $CD8\alpha^-$ DC have not been as well characterized as the $CD8\alpha^+$ DC subset, though this may be due to the possibility that there are multiple DC subsets represented within the $CD8\alpha^-$ DC population. However, it has been shown that $CD8\alpha^-$ DCs are not efficient at cross presentation, but may be more effective at the generation of a $CD4^+$ T cell response.

Since they are the gatekeepers to productive adaptive immunity, dendritic cells have a tightly regulated maturation program, constantly sampling their environments for foreign molecules, and when activated, initiating a molecular program that specializes them for T cell activation in response to a pathogen. Given the functional differences associated with different dendritic cell subsets, as well as the functional differences associated with other potential antigen presenting cells, set out to determine if *Listeria* and Vaccinia vaccinations utilized the same or different populations of primary antigen presenting cells. If different populations of professional APCs were responsible for priming $CD8^+$ T cell responses to these two vaccinations, this could potentially explain the different expression of PD-1 on the surface of these $CD8^+$ T cells, as well as the difference in anti-tumor immunity. However, if the same population of professional APCs were responsible for the priming of $CD8^+$ T cell responses, then the programming of those APCs and the subsequent activation of the $CD8^+$ T cells must be different due to recognition of the different vectors by the professional antigen presenting cells.

Chapter Specific Materials and Methods

Direct ex vivo Antigen Detection (DEAD) Assay With Experienced or Naïve T Cells

Direct *ex vivo* antigen detection assays with antigen experienced cells were performed as previously described, with one key exception. For the presenting population, populations of professional antigen presenting cells from the spleens of host mice were combined and depleted of $CD19^+$ B and $CD3\varepsilon^+$ T cells using magnetic bead enrichments and sorted using the following identification markers: B cells: $CD3\varepsilon^-CD19^+DX5^-$, Granulocytic Myeloid Cells: $CD3\varepsilon^-CD19^-DX5^-CD11B^{hi}Ly6G^{hi}Ly6C^{int}$, Monocytic Myeloid Cells: $CD3\varepsilon^-CD19^-DX5^-CD11B^{hi}Ly6G^-Ly6C^{hi}$, $CD8\alpha^-$ Dendritic Cells: $CD3\varepsilon^-CD19^-DX5^-CD11C^{hi}CD8\alpha^-$, and $CD8\alpha^+$ Dendritic Cells: $CD3\varepsilon^-CD19^-DX5^-CD11C^{hi}CD8\alpha^+$. For the B cell population, a small fraction of the initial splenocyte population was left undepleted prior to sorting. Following FACS sorting, populations were recounted. Cells were plated in complete RPMI in a 96 well round bottom plate with $2x10^5$ presenting cells and $2x10^4$ CFSE labeled $CD8^+$ OT-1 T cells for six hours. After the first six hour incubation, protein transport blockade cocktail was added, and an additional 6 hours of culture was used to restimulate the cells prior to staining for IFNγ.

For stimulation of naïve T cell populations, the same process as above described was used to isolate the sort the presenting populations. However, naïve OT-1 T cells, just isolated and enriched by magnetic bead selection, were CFSE labeled, and co-cultured with presenters at the above ratios in the presence of 2 ng/mL mIL-2 for 72 hours. After 3 days in culture, cells were examined for CFSE dilutions, and checkpoint protein expression by flow cytometry.

RNA Extraction, Microarray Generation, and Microarray Analysis

For the generation of microarray data, host mice were vaccinated 24 hours prior to harvest with either PBS, $1x10^6$ pfu Vaccinia-OVA, or $1x10^7$ *Listeria*-OVA via tail vein injection. As shown previously (Fig 4-1, 4-3. and 5-1) these vaccine doses result in roughly equivalent division of cognate antigen-specific T cells in vivo. Spleens were harvested as previously described, and B and T cells were depleted by magnetic bead selection, as described for the DEAD assay. $CD8\alpha^+$ dendritic cells were sorted with the same sorting strategy previously shown. Following sorting, RNA extraction was performed using a RNEASY Micro kit (Qiagen, San Diego, CA) following the manufacturer's instructions, and including homogenization with a Qiagen cell shredder(Qiagen, San Diego, CA). RNA was eluted from the column, and flash frozen using dry ice and liquid nitrogen, before being submitted to the JHU Deep Sequencing and Microarray Core Facility. RNA was amplified using Nugene technology, and run on an Affymetrix Mouse Gene 2.0ST chip (Affymetrix, Santa Clara, CA). Hybridization signal was harvested using an Affymetrix Gene Chip system. Intensity data was converted into fold change, and P values calculated using Partek analysis software (Partek, Singapore). Figures and tables were generated using Spotfire analysis software (Tibco, Boston, MA).

Multi-Target Co-Culture Assay

For the modified transwell assay experiments, antigen presenting dendritic cells were generated and harvested as seen previously for the DEAD assay experiments. Briefly, animals were vaccinated 24 hours prior to harvest (with either $1x10^6$ Vaccinia-

HA or $1x10^7$ *Listeria*-OVA), spleens were pooled, RBCs lysed, and B and T cells were depleted prior to sorting $CD8\alpha^+$ dendritic cells as seen previously. $CD8^+$ OT-I T cells and $CD8^+$ Clone 4 T cells were magnetically isolated and CFSE labeled as seen previously. T cells populations were mixed such that $2x10^4$ of both OT-I and Clone 4 T cells were present in each well of the 96 well plate. Presenting $CD8\alpha^+$ dendritic cells were added as described, either alone or mixed, along with 2ng/mL mIL-2 (Figure 7-8) (Peprotech, Rocky Hill, NJ). After 72 hours, cells were stained for markers of interest, and run on a LSR II instrument.

Results

Listeria-OVA Vaccine Associated Antigens Are Presented Exclusively By Monocytic Myeloid Cells and $CD8\alpha^+$ Dendritic Cells

To test the hypothesis that *Listeria*-OVA and Vaccinia-OVA associated antigens were being presented by two different populations of professional antigen presenting cells, we had to first determine the populations of cells capable of presenting *Listeria*-OVA associated antigens within the context of MHC I. To explore the different populations of possible professional antigen presenting cells, we utilized a DEAD assay similar to the previous studies, but with FACS sorted populations of professional antigen presenting cells. After reviewing markers of different professional antigen presenting cells in the literature, we designed a FACs sorting panel to isolate and sort to >97% purity six different possible populations of potential professional antigen presenting cells; namely, B cells, monocytic myeloid cells, granulocytic myeloid cells, $Ly6C^-$ $Ly6G^-$ myeloid cells, CD8α- dendritic cells, and CD8α+ dendritic cells (Fig 7-1). Following

FACS sorting, we co-cultured these cells with previously activated OT-I T cells, and looked for IFNγ production as a method of determining expression of OVA in the context of MHC I. Surprisingly, despite the variety of populations available for infection, *Listeria*-OVA associated antigens were only presented to $CD8^+$ T cells by the monocytic myeloid cells and the $CD8\alpha^+$ dendritic cells (Fig 7-2a,b). These data suggest that, despite the large number of possible antigen presenting cells, vaccinations *in vivo* are likely being targeted to a small number of professional antigen presenting cells.

Listeria Monocytogenes Is Found In $CD8\alpha^+$ Dendritic Cells Early During the Immune Response, Followed By Later Localization In Monocytic Myeloid Cells

To confirm the findings from the DEAD assay, we wanted to examine whether *Listeria* could be found in one or both of the populations previously identified. To do this, we utilized a *Listeria Monocytogenes* vector which had been genetically modified to express green fluorescent protein (GFP), and examined the different populations of antigen presenting cells. As can be seen, GFP expression was only found in the monocytic myeloid and $CD8\alpha^+$ dendritic cell populations during the time assayed. However, the kinetics of uptake were different between the two populations, with *Listeria*-GFP being found initially in the $CD8\alpha^+$ dendritic cell population as early as 60 minutes following infection (Fig 7-3). GFP signal was not detected in the monocytic myeloid population until 24 hours, suggesting that these two populations have different capabilities for either infection or phagocytic uptake of the attenuated *Listeria Monocytogenes* vector. Surprisingly, only a small fraction of either population was seen to be GFP positive in this assay, further suggesting that only a small subset of

professional antigen presenting cells were being activated by these vaccines, and that these cells have an extremely high functional capability for the activation of $CD8^+$ T cells.

Naïve $CD8^+$ T Cells Are Primed by $CD8\alpha^+$ Dendritic Cells In Response To Both Listeria and Vaccinia Based Vaccinations

While OVA peptide was being presented to experienced $CD8^+$ T cells by both monocytic myeloid cells and CD8α+ dendritic cells, priming a naïve $CD8^+$ T cell response requires multiple signals, including co-stimulation and cytokine production. Therefore, we wanted to test whether naïve $CD8^+$ T cells could be primed *in vitro* by the professional antigen presenting populations previously examined. Furthermore, we wanted to know which professional antigen presenting population was primarily responsible for priming $CD8^+$ T cell responses during Vaccinia-OVA vaccination. Using the same FACS sorting panel as previously described, we co-cultured these cells with CFSE labeled, naïve $CD8^+$ T cells. In this system, we found that $CD8\alpha^+$ dendritic cells were the most robust stimulators of naïve $CD8^+$ T cell responses for either *Listeria* or Vaccinia vaccination (Fig 7-4a,b). Interestingly, when co-cultured with $CD8\alpha^+$ dendritic cells from a CAG-OVA mouse, naïve $CD8^+$ OT-I T cells were stimulated to divide by all the professional antigen presenting populations, showing that each of these populations can prime naïve OT-1s if OVA peptide is being presented in the context of MHC I. We also saw a small amount of naïve $CD8^+$ T cell priming by the monocytic myeloid cells when vaccinated with *Listeria*-OVA, which is in line with the presentation of OVA by these cells in the previous assay. However, since the monocytic myeloid cells were far

less potent than the CD8α$^+$ dendritic cells, we believe that the most biologically significant population of professional antigen presenting cells for both *Listeria* and Vaccinia vaccinations is the CD8α$^+$ dendritic cell population. These data suggest that the differences in CD8$^+$ T cell expression of checkpoint proteins was not due to the utilization of different professional antigen presenting cell populations.

CD8α$^+$ Dendritic Cells From Listeria Vaccinated Mice Are Sufficient For CD8$^+$ T Cell Activation Without PD-1 Up Regulation

While the CD8α$^+$ dendritic cells FACS sorted from vaccinated mice were capable of stimulating naïve CD8$^+$ T cells to divide, it was still possible that other innate immune cells or soluble factors were responsible for the CD8$^+$ T cell activation without PD-1 up regulation *in vivo*. Therefore, we wanted to examine the capability of CD8α$^+$ dendritic cells from vaccinated mice to regulate PD-1 expression on CD8$^+$ T cells during activation. Naïve OT-1s co-cultured with CD8α$^+$ dendritic cells from a CAG-OVA host divided in response to their antigen, as seen previously, but expressed large amounts of PD-1. However, despite utilizing the same professional antigen presenting cell population, *Listeria*-OVA vaccinated CD8α$^+$ dendritic cells were sufficient to activate naïve OT-1s without allowing PD-1 up regulation, while co-culture with Vaccinia-OVA vaccinated CD8α$^+$ dendritic cells resulted for similar expression of PD-1 as seen in the *in vivo* adoptive transfer studies (Fig 7-5). Interestingly, naïve OT-1s activated *in vitro* by CD8α$^+$ dendritic cells from a host animal receiving *Listeria*-OVA vaccination may express even less PD-1 than was seen in the *in vivo* adoptive transfer studies, suggesting that this interaction is responsible for the *in vivo* phenotype. Furthermore, these data

show that there is a protein capable of restricting PD-1 expression that is present on or secreted by CD8α^+ dendritic cells when they are vaccinated with this live-attenuated *Listeria* vaccine.

Listeria and Vaccinia Vaccinations Generate Two Completely Different Transcriptional Programs in CD8α^+ Dendritic Cells

In order to determine whether CD8α^+ dendritic cells respond differently to *Listeria* and Vaccinia vaccinations, we examined the transcriptional profile of *Listeria* or Vaccinia vaccinated CD8α^+ dendritic cells by microarray analysis. FACS sorting was performed as previously described, and post sort analysis showed that the CD8α^+ dendritic cell populations that were analyzed by microarray were >97% pure (Fig 7-6a). Surprisingly, the differences in the transcriptional profile were clear even before the application of a significance filter, as the principal component analysis displayed very clear groupings (Fig 7-6b). From the global perspective, the transcriptional program being expressed in *Listeria* vaccinated CD8α^+ dendritic cells was extremely different from the program activated in response to Vaccinia, or that expressed in resting CD8α^+ dendritic cells (Fig 7-7). These data suggest that, despite both vaccines activating the same professional antigen presenting cell population, the response of the CD8α^+ dendritic cell population to these two vaccines is incredibly different, and likely the cause of the differences in PD-1 expression, as well as the differences in the generation of anti-tumor immunity.

At the individual gene level, *Listeria* vaccinated CD8α^+ dendritic cells expressed higher levels of several dendritic cell maturation markers, including CD80, CD86, and

CD70, though surprisingly, no difference in CD40 expression was seen (Table 7-1). Furthermore, transcripts for the intracellular sensors STING and RIGI were detected, as well as several chemokines and cytokines. We also saw increased levels of the transcription factor CISH, which has been correlated with dendritic cell maturation status[172]. In terms of genes down regulated, one gene in particular stood out. Rgs18 is a GTPase activating protein (GAP), which prevents G protein coupled signaling by hydrolyzing GTP to GDP quickly. *Listeria* vaccination resulted in significant down regulation of this gene when compared either to resting cells or to Vaccinia vaccination, suggesting that this protein may have a role in the maintenance of tolerance in the steady state of resting (or sub-optimally activated) dendritic cells (Table 7-2). Together, these transcripts identify a different, and potentially more potent, dendritic cell activation program than that of dendritic cells stimulated by vaccination with *Vaccinia*.

CD8α^+ Dendritic Cells From Listeria Vaccinated Mice Modulate PD-1 Expression During Naïve CD8$^+$ T Cell Activation In A Cell Contact Dependent Manner

Since the list of differentially expressed genes is rather large, we sought to determine if control of PD-1 expression on CD8$^+$ T cells during activation is a function of soluble factors being secreted by *Listeria* vaccinated CD8α^+ dendritic cells, or if direct contact between cognate T cells and antigen presenting DC is required. To test this, we took advantage of the previous experiments, where we saw that *Listeria* vaccination was capable of activating either Clone 4 or OT-I CD8$^+$ T cells without substantial PD-1 up regulation, despite their differing genetic backgrounds and MHC I alleles (Figure 5-1, 5-5). Therefore, we designed and performed a modified transwell assay, where we co-cultured *Listeria*-OVA vaccinated CD8α^+ dendritic cells (C57/B6) and/or *Vaccinia*-HA

vaccinated CD8α^+ dendritic cells (B10.D2) in the same well as CFSE labeled OT-I and Clone 4 CD8$^+$ T cells (Fig 7-8a). In this system, CD8$^+$ T cells would only interact and form a cell-contact dependent immune synapse with their cognate CD8α^+ dendritic cell population. However, both populations would be in culture together, so if there were soluble factors either promoting or suppressing expression of PD-1, these soluble factors would be able to affect both CD8$^+$ T cell populations (Fig 7-8a). As in previous studies, when both CD8$^+$ T cell populations were co-cultured with only *Vaccinia*-HA vaccinated CD8α^+ dendritic cells, only the Clone 4 population divided, and expressed intermediate levels of PD-1. Furthermore, when both CD8$^+$ T cell populations were co-cultured with *Listeria*-OVA vaccinated CD8α^+ dendritic cells, only the OT-I population was stimulated to divide, and did not express PD-1 as previously demonstrated (Fig 7-8b). Surprisingly, when both populations of vaccinated CD8α^+ dendritic cells were co-cultured with both populations of CD8$^+$ T cells, both populations divided, and acted as if only their cognate CD8α^+ dendritic cell population was present. Together, these data show that soluble factors are not the determining factor for PD-1 expression in this system, and that instead, some signaling pathway that requires cell to cell contact between the CD8α^+ dendritic cells and the responding CD8$^+$ T cell is determining the expression of PD-1 in response to these two vaccinations.

Summary

Traditionally, it is understood that the generation of an adaptive immune response to a pathogen or vaccination requires that professional antigen presenting cells recognize the foreign material, mature in response to that stimulus, and subsequently prime naïve

$CD8^+$ T cell responses to that pathogen. However, recent work has begun to identify different subpopulations of antigen presenting cells, such as the several different populations of dendritic cells that have been established. These studies have clearly show heterogeneity of response within a specific subpopulation of dendritic cells, dependent on the vaccine vector being recognized. In this chapter we have shown that both *Listeria Monocytogenes* and Vaccinia Virus are presented to the adaptive immune system by the same subset of professional antigen presenting cells, namely $CD8\alpha^+$ dendritic cells. This population is known for its potent cross presentation function, as well as its potential role in the presentation of tumor associated antigens, making a vaccine that activates this subset of cells a very attractive vector. Yet, despite being presented by the same population, we have found that manner in which the $CD8\alpha^+$ dendritic cells stimulate a $CD8^+$ T cell response is different depending on the vaccine they have recognized. *In vitro*, co-culture of Vaccinia or *Listeria* vaccinated $CD8\alpha^+$ dendritic cells with naïve $CD8^+$ T cells was sufficient to replicate the *in vivo* expression levels of PD-1. Furthermore, *Listeria* and Vaccinia vaccinations resulted in completely different transcriptional profiles in the CD8α+ dendritic cells, suggesting that this population of professional antigen presenting cells is capable of discerning between multiple types of pathogens, and responding appropriately. Finally, we found that $CD8\alpha^+$ dendritic cells do not modulate PD-1 expression on $CD8^+$ T cells in our model through the use of soluble factors. This implies that there is a cell surface signaling pathway between $CD8\alpha^+$ dendritic cells and naïve $CD8^+$ T cells that is capable of modulating PD-1 expression on the responding cells. This could be another attractive mechanism for clinical intervention,

given the clinical results seen with the use of anti PD-1 alone and in combination with other agents.

Figure 7-1

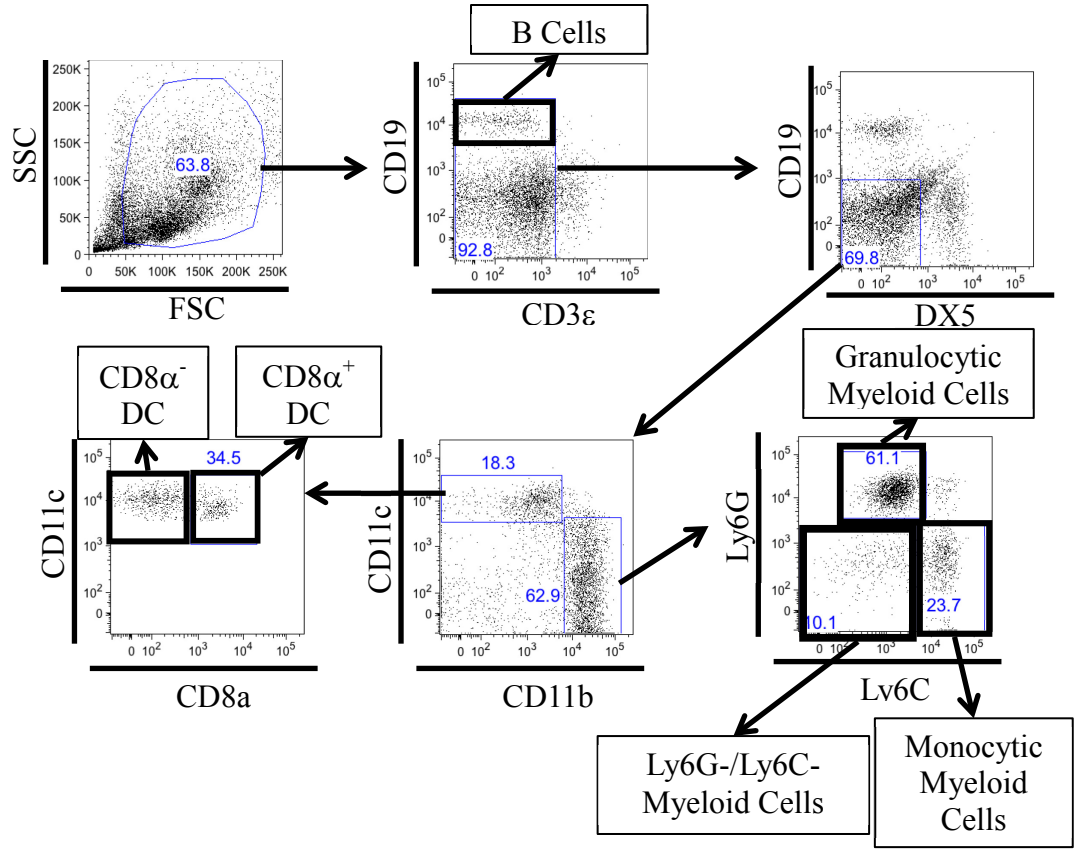
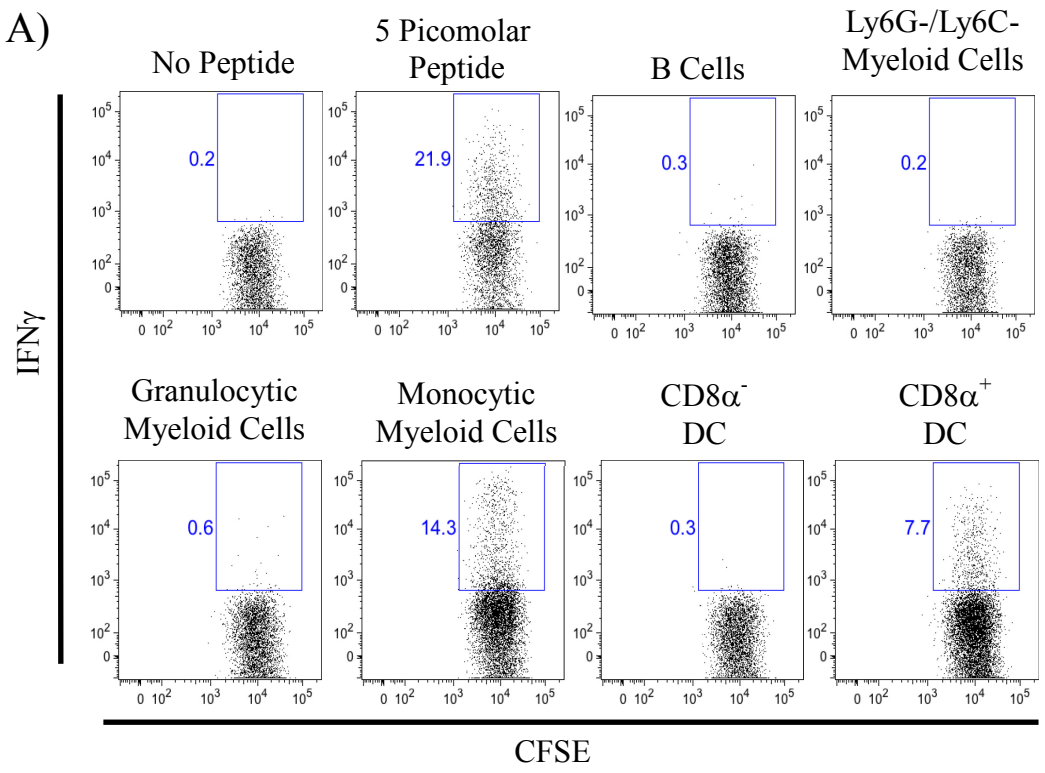


Figure 7-1: Sorting Strategy for the Identification and Isolation of Various Professional Antigen Presenting Cell Populations

To isolate different potential professional antigen presenting cell populations, cells were stained with a variety of extracellular markers and FACS sorted. Prior to sorting, splenocytes were depleted of $CD3\varepsilon^+$ and $CD19^+$ cells through the use of magnet bead enrichment. The different populations of antigen presenting cells were defined and named as follows: B cells: $CD3\varepsilon^-CD19^+DX5^-$, Granulocytic Myeloid Cells: $CD3\varepsilon^-CD19^-DX5^-CD11B^{hi}Ly6G^{hi}Ly6C^{int}$, Monocytic Myeloid Cells: $CD3\varepsilon^-CD19^-DX5^-CD11B^{hi}Ly6G^-Ly6C^{hi}$, $CD8\alpha^-$ Dendritic Cells: $CD3\varepsilon^-CD19^-DX5^-CD11C^{hi}CD8\alpha^-$, and $CD8\alpha^+$ Dendritic Cells: $CD3\varepsilon^-CD19^-DX5^-CD11C^{hi}CD8\alpha^+$.

Figure 7-2


A)
No Peptide
5 Picomolar Peptide
B Cells
Ly6G-/Ly6C- Myeloid Cells
0.2
21.9
0.3
0.2
Granulocytic Myeloid Cells
Monocytic Myeloid Cells
CD8α- DC
CD8α+ DC
0.6
14.3
0.3
7.7
IFNγ
CFSE

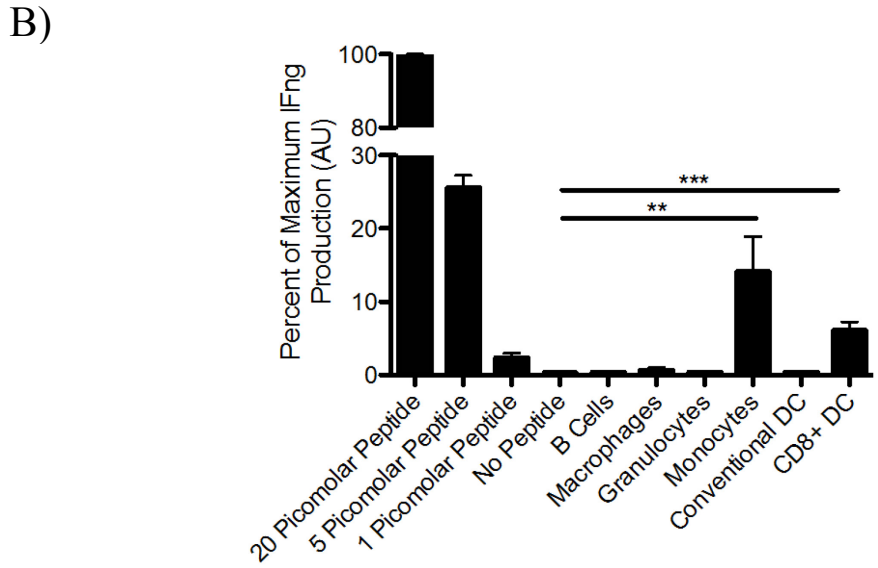

B)
Percent of Maximum IFng Production (AU)
100
80
30
20
10
0
***
**
20 Picomolar Peptide
5 Picomolar Peptide
1 Picomolar Peptide
No Peptide
B Cells
Macrophages
Granulocytes
Monocytes
Conventional DC
CD8+ DC

Figure 7-2: *Listeria* Vaccination Associated Antigens Are Presented Exclusively on Monocytic Myeloid Cells and CD8α$^+$ Dendritic Cells

Populations of professional antigen presenting cells were sorted as previously described 24 hours after intravenous vaccination with *Listeria*-OVA or PBS for control, peptide pulsed splenocytes. Following isolation of individual populations, presenting cells were co-cultured with previously activated, CFSE labeled OT-I CD8$^+$ T cells for six hours before protein transport blockade was added, and cultured for additional six hours with protein transport blockade before being stained for IFNγ production. Peptide pulsed splenocytes were used as a positive control for peptide presentation. A) Dot plots depicting typical IFNγ responses and B) IFNγ production quantified and normalized to IFNγ production in response to 20 picomolar pulsed splenocytes. Data shown are representative of at least 2 independent experiments, with duplicate or triplicate wells being cultured depending on the number of cells available.

Figure 7-3

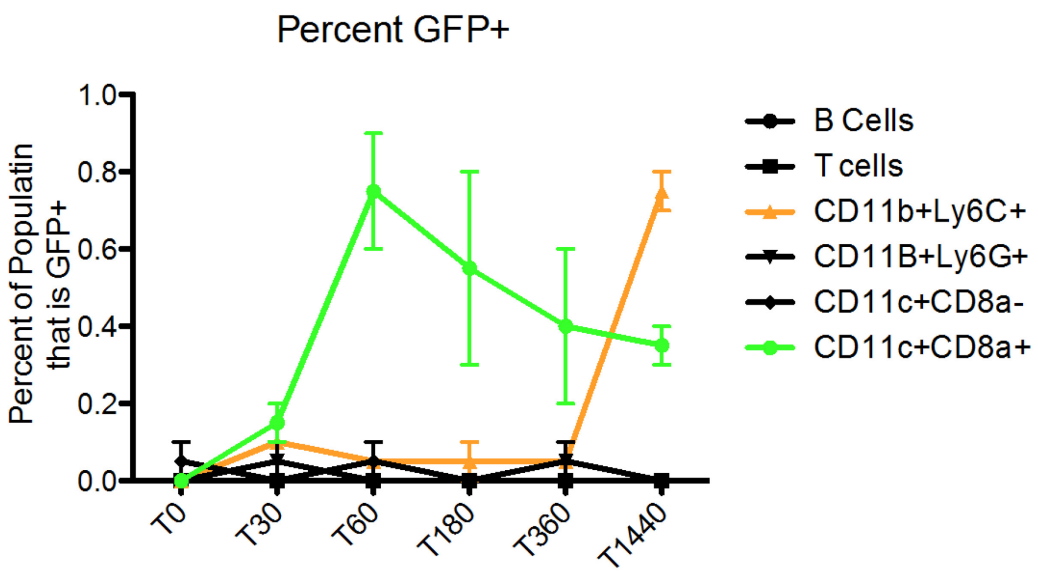
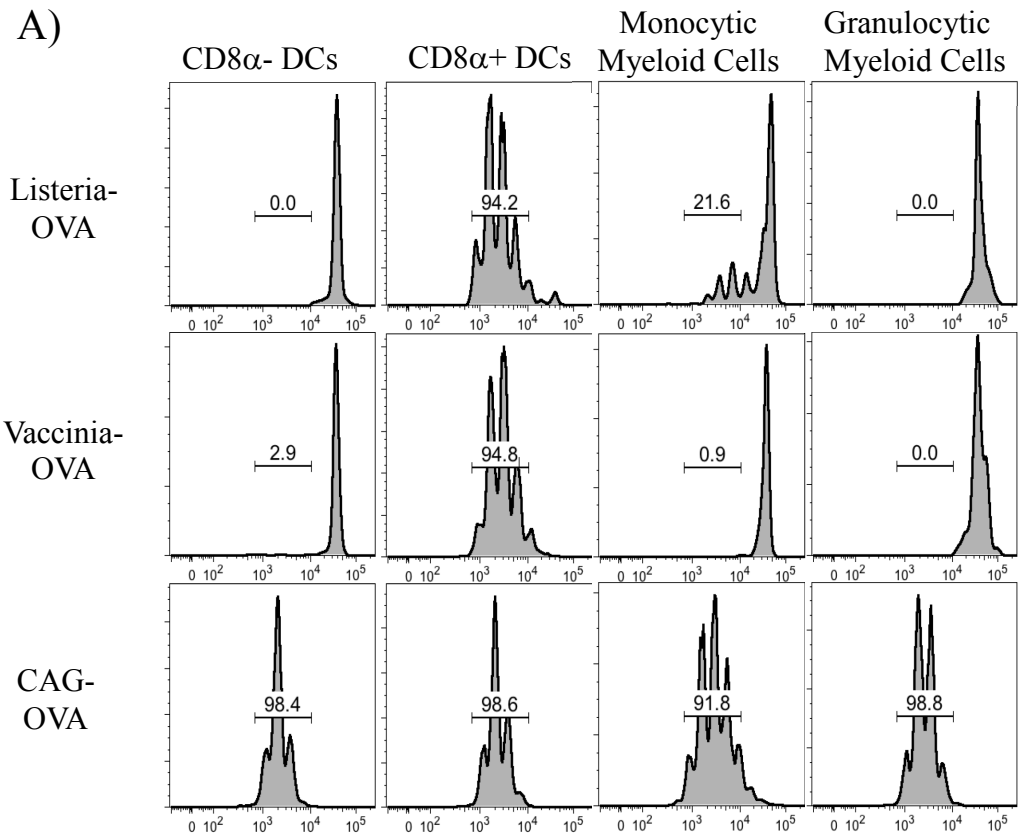


Figure 7-3: *Listeria* is Detected in Different Populations of Professional Antigen Presenting Cells with Unique Kinetics

Host mice were injected with *Listeria*-GFP and splenocytes were examined at the indicated time points for GFP expression by flow cytometry. Graph depicts percent of the labeled population that expressed GFP. Data shown are representative of at least 2 independent experiments, with n=3-4 animals per time point.

Figure 7-4


A)
CD8α- DCs
CD8α+ DCs
Monocytic Myeloid Cells
Granulocytic Myeloid Cells
Listeria-OVA
Vaccinia-OVA
CAG-OVA
0.0
94.2
21.6
0.0
2.9
94.8
0.9
0.0
98.4
98.6
91.8
98.8

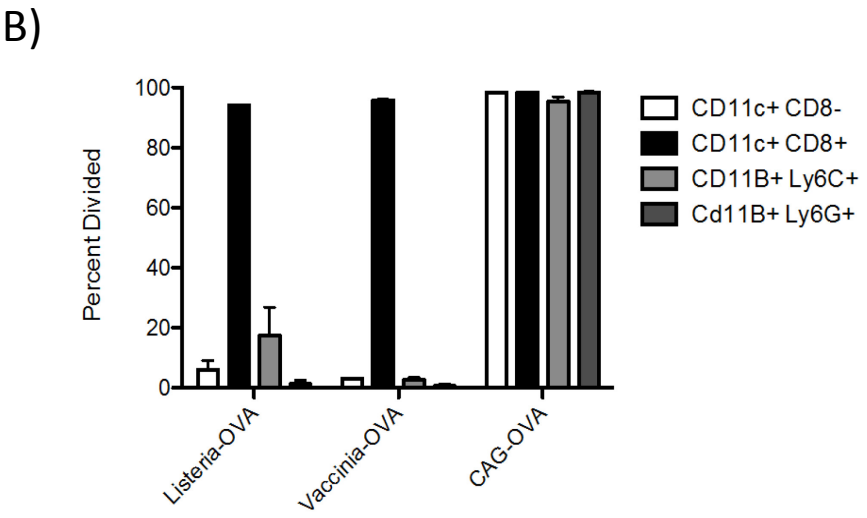
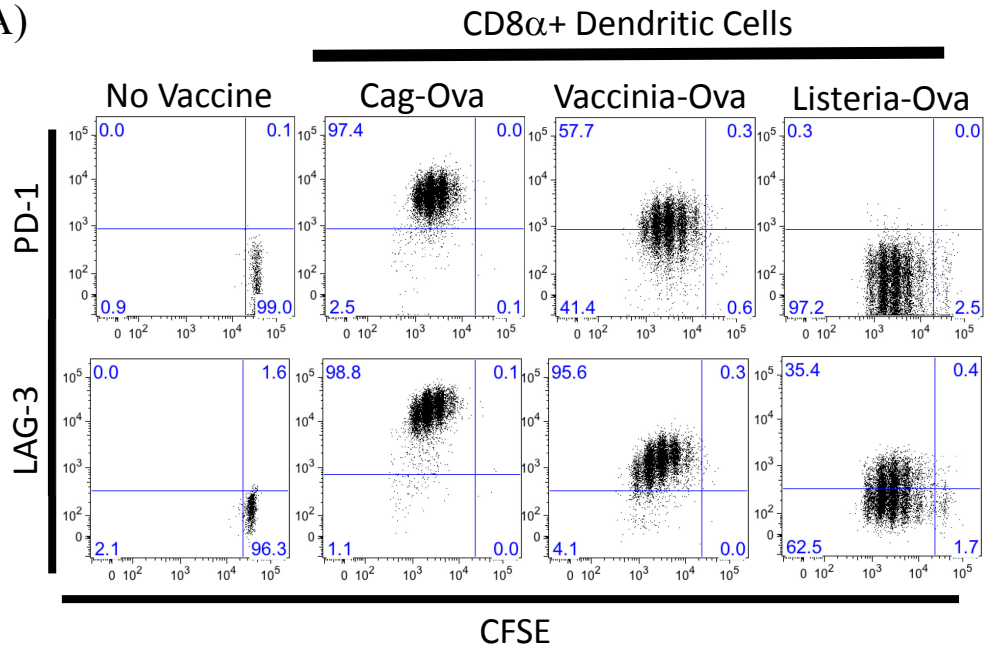

B)
Percent Divided
100
80
60
40
20
0
Listeria-OVA
Vaccinia-OVA
CAG-OVA
CD11c+ CD8-
CD11c+ CD8+
CD11B+ Ly6C+
Cd11B+ Ly6G+

Figure 7-4: **CD8α$^+$ Dendritic Cells Are the Primary Professional Antigen Presenting Cell Responsible for Priming CD8$^+$ T cells Responses to *Listeria* and Vaccinia Vaccines**

Populations of professional antigen presenting cells were sorted from congenically marked CD45.1 mice as previously described, 24 hours after intravenous vaccination with *Listeria*-OVA or Vaccinia-OVA. Unvaccinated CAG-OVA splenocytes were also harvested as positive controls for presentation of OVA peptide. Following isolation individual populations, presenting cells were co-cultured with naïve, CFSE labeled OT-I CD8$^+$ T cells for 72 hours with 2ng/mL mIL-2 before being examined for division in response to a presenting population. A) Histogram analysis of dividing cells and B) quantification of replicate experiments. Data shown are representative of at least 3 independent experiments, with triplicate wells being used in each condition.

Figure 7-5

A)


CD8α+ Dendritic Cells
No Vaccine
Cag-Ova
Vaccinia-Ova
Listeria-Ova
PD-1
LAG-3
0.0
0.1
0.9
99.0
97.4
0.0
2.5
0.1
57.7
0.3
41.4
0.6
0.3
0.0
97.2
2.5
0.0
1.6
2.1
96.3
98.8
0.1
1.1
0.0
95.6
0.3
4.1
0.0
35.4
0.4
62.5
1.7
CFSE

B)

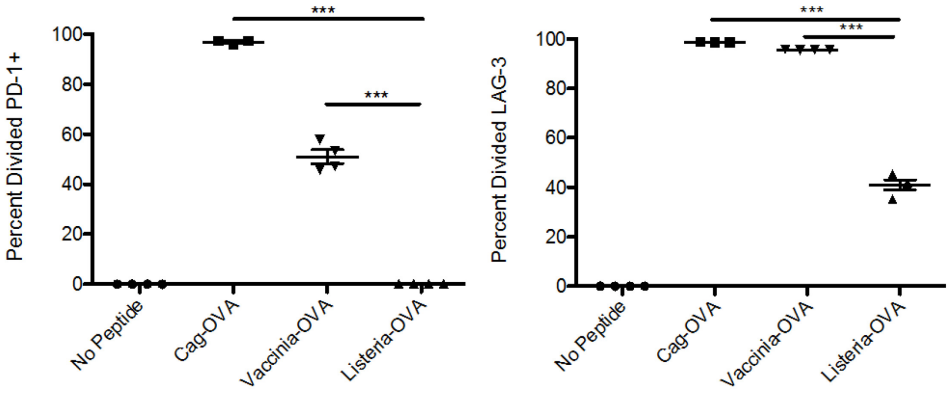
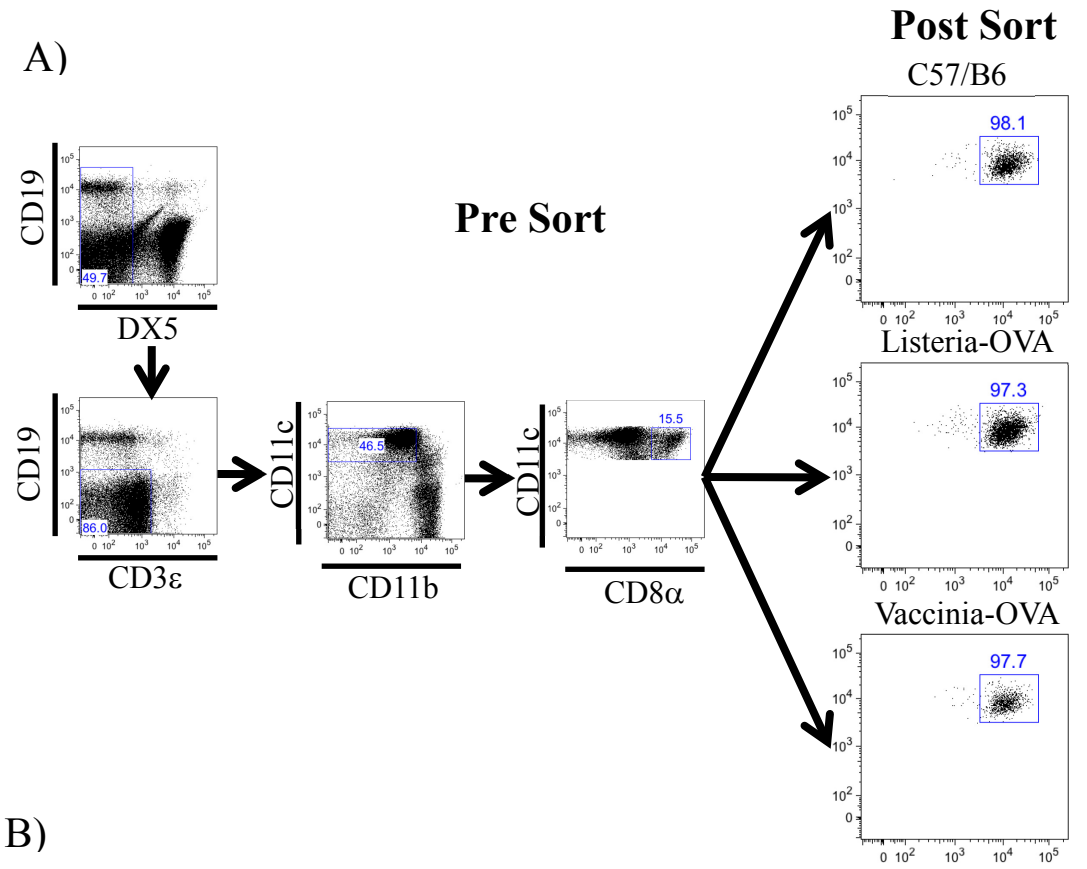

Percent Divided PD-1+
Percent Divided LAG-3
***
***
***
***
100
80
60
40
20
0
No Peptide
Cag-OVA
Vaccinia-OVA
Listeria-OVA

Figure 7-5: **Vaccinated CD8α$^+$ Dendritic Cells Are Sufficient To Replicate the *in vivo* Checkpoint Expression Levels *in vitro***

Populations of professional antigen presenting cells were sorted from congenically marked CD45.1 mice as previously described, 24 hours after intravenous vaccination with *Listeria*-OVA or Vaccinia-OVA. Unvaccinated CAG-OVA splenocytes were also harvested as positive controls for presentation of OVA peptide. Following isolation individual populations, presenting cells were co-cultured with naïve, CFSE labeled OT-I CD8$^+$ T cells for 72 hours before being examined for division and checkpoint expression in response to a presenting population. A) Dot plots of PD-1 and LAG-3 expression and B) quantification of replicate wells. Data shown are representative of at least 3 independent experiments, with triplicate wells being used in each condition.

Figure 7-6


A)
Post Sort
C57/B6
Pre Sort
CD19
49.7
DX5
CD19
86.0
CD3ε
CD11c
46.5
CD11b
CD11c
15.5
CD8α
98.1
Listeria-OVA
97.3
Vaccinia-OVA
97.7

B)

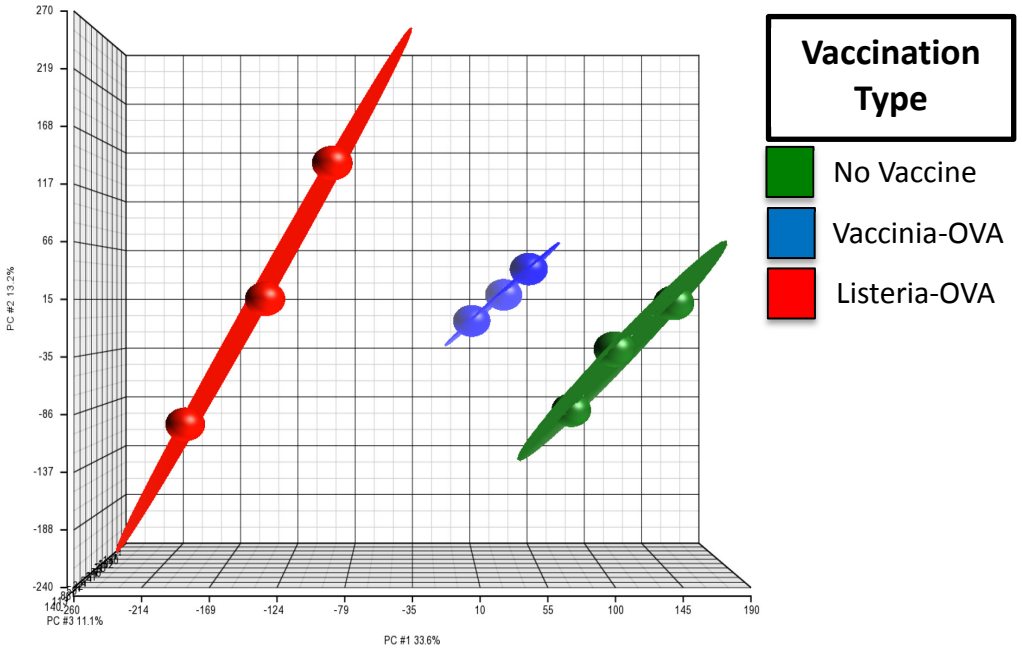

Vaccination Type
No Vaccine
Vaccinia-OVA
Listeria-OVA
PC #2 13.2%
PC #3 11.1%
PC #1 33.6%

Figure 7-6: **Sorting Strategy and Quality Control Analysis of Microarray Results**

24 hours prior to sorting, C57/B6 mice were vaccinated with *Listeria*-OVA, Vaccinia-OVA, or PBS. Splenocytes were harvested and depleted of T cells and B cells before sorting through the use of magnetic bead isolation kits. To ensure appropriate cell number, mice from each group were divided into groups of 4, and processed and sorted together as one replicate. A) FACs sorting strategy and post sort analysis for purity. B) Principal component analysis on generated microarray data.

Figure 7-7

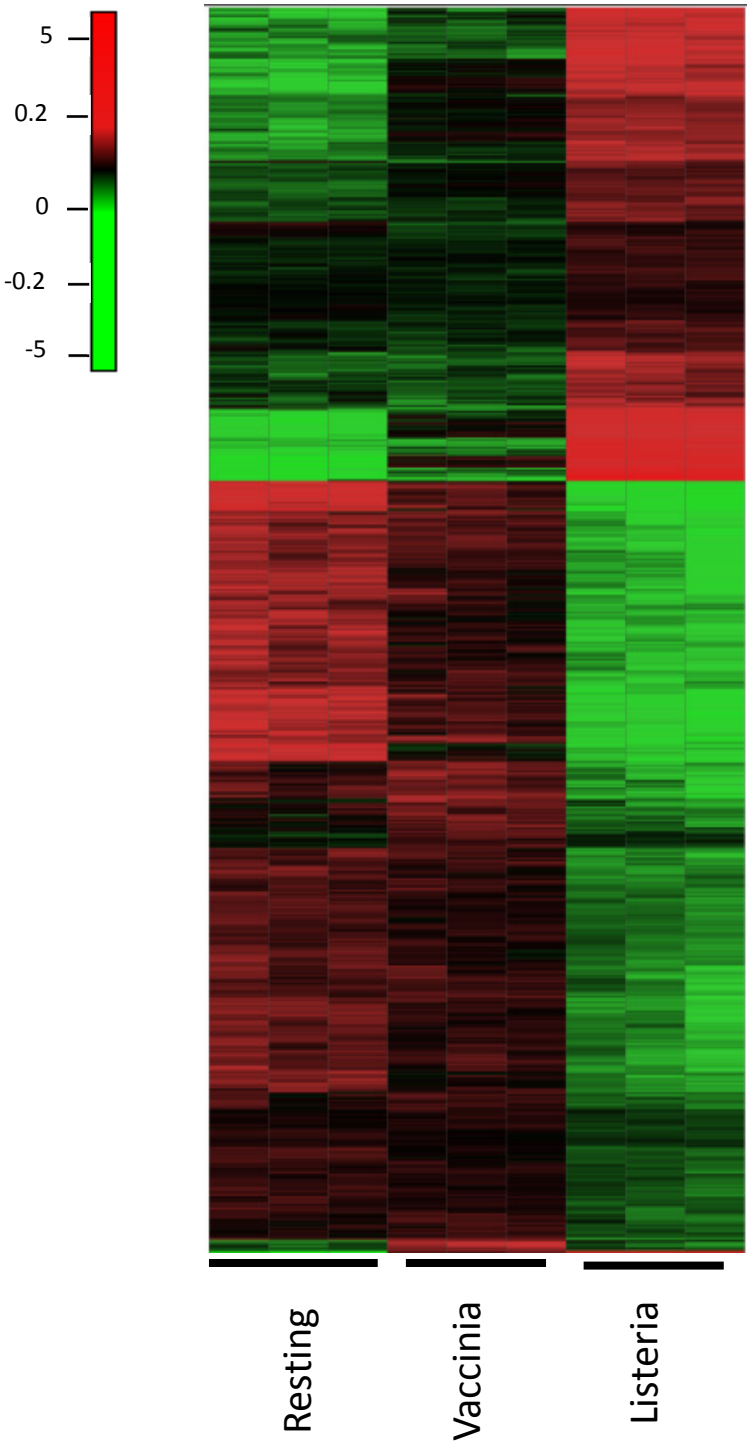


Figure 7-7: **CD8α^+ Dendritic Cells Activate Significantly Different Transcriptional Programs in Response to *Listeria* or Vaccinia Vaccination**

C57/B6 mice were vaccinated with *Listeria*-OVA, Vaccinia-OVA, or PBS. Twenty four hours later, mice were pooled, CD8α^+ dendritic cells were FACS sorted, and total RNA was extracted. Microarray analysis was performed using an Affymetrix gene 2.0 chip, and heat maps were generated using Spotfire analysis software. Heat map depicted is filtered on genes with a false discovery rate p value of less than 0.05 when comparing *Listeria* and Vaccinia vaccinated CD8α^+ dendritic cells.

Figure 7-8

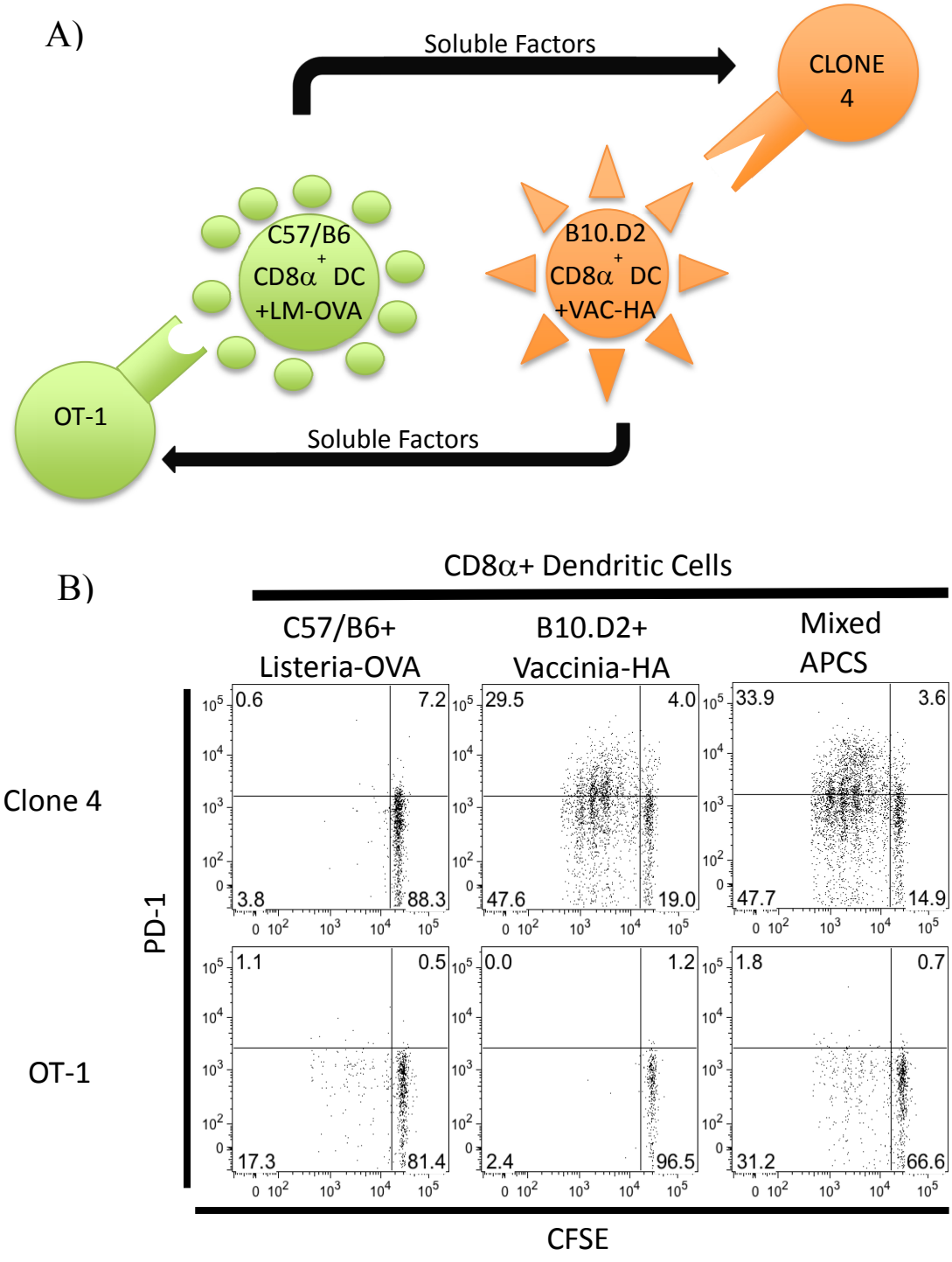

A)
Soluble Factors
CLONE 4
C57/B6 CD8α+ DC +LM-OVA
B10.D2 CD8α+ DC +VAC-HA
OT-1
Soluble Factors
B)
CD8α+ Dendritic Cells
C57/B6+ Listeria-OVA
B10.D2+ Vaccinia-HA
Mixed APCS
Clone 4
OT-1
PD-1
CFSE
0.6
7.2
3.8
88.3
29.5
4.0
47.6
19.0
33.9
3.6
47.7
14.9
1.1
0.5
17.3
81.4
0.0
1.2
2.4
96.5
1.8
0.7
31.2
66.6

Figure 7-8: Regulation of the Expression of PD-1 on $CD8^+$ T Cells During Activation By $CD8\alpha^+$ Dendritic Cells Is Not Mediated By Soluble Factors

$CD45.1^+$ C57/B6 mice were vaccinated with *Listeria*-OVA while $Thy1.2^+$ B10.D2 mice were vaccinated with Vaccinia-HA. 24 hours later, $CD8\alpha^+$ dendritic cells were isolated as previously described. $Thy1.1^+$ Clone 4 and $CD45.2^+$ OT-1 $CD8^+$ T cells were isolated by magnetic bead enrichment, CFSE labeled, and mixed together at a 1:1 ratio. $4x10^4$ total $CD8^+$ T cells were co-cultured with $2x10^5$ *Listeria*-OVA vaccinated C57/B6 $CD8\alpha^+$ dendritic cells, $2x10^5$ Vaccinia-HA vaccinated C57/B6 $CD8\alpha^+$ dendritic cells, or a mixture of $2x10^5$ of both populations, for a total of $4x10^5$ dendritic cells in the mixed culture. After 72 hours, expression of PD-1 on the two populations of T cells was examined. A) A model depicting the potential for soluble factors to interact with both populations of T cells, while cell surface factors will be background restricted. B) Flow cytometric analysis of PD-1 expression, gated on CD8, CFSE, and the proper congenic markers. Data shown are representative of at least 3 independent experiments, with triplicate wells being used in each condition.

Table 7-1

Up Regulated By Listeria Relative to Vaccinia Vaccinia

Gene Symbol	Fold-Change	FDR P Value
S1pr3	21.1906	0.0012064
Gm4934	21.0706	0.00211307
Pla1a	17.0982	0.000435575
Cxcr5	16.7979	0.00041528
S100a8	16.1519	0.00459743
Cish	16.1093	0.00325763
Ramp3	15.3529	0.000375625
Npr1	15.2864	0.00135258
Slc27a3	15.1266	0.00041528
Cnn3	13.5664	0.00156995
Flrt3	11.7063	0.00137052
F830016B08Rik	11.3015	0.000706254
Il1rn	10.7128	0.00137052
Ndrg2	10.443	0.00063002
Il6	10.0829	0.00667287
Slc44a5	9.97064	0.00157941
AA467197	9.71818	0.000375625
Gm4951	9.50768	0.00645207
Npr1	8.99947	0.00063002
Ikzf4	8.79949	0.00135258
Trim30c	8.40917	0.0050555
Cd70	8.1016	0.00488802
Cish	7.97554	0.00744893
Olfr1396	7.69177	0.00183456
Osm	7.60459	0.000706254
Ubd	7.38177	0.00137052
Il12rb2	7.19496	6.58E-05

Table 7-1: Top Genes Up Regulated in Response to *Listeria* Vaccination in CD8α^+ Dendritic Cells

Using Partek and Spotfire analysis software, transcript expression level was calculated as fold change vs. Vaccinia vaccinated CD8α^+ dendritic cell expression levels.

Table 7-2

Down Regulated By Listeria Relative to Vaccinia

Gene Symbol	Fold-Change	FDR P Value
Rgs18	-7.46666	0.00255549
Arhgef6	-6.6257	0.000951948
Serpinb10	-6.49823	0.00472494
Cxx1c	-6.05146	0.0012064
H2-M2	-6.03532	0.00041528
Anxa1	-5.7341	0.00186397
Arsb	-5.56749	0.00211307
Slc9a9	-5.36468	0.00313263
Klra17	-5.35277	0.0281839
Stap1	-5.28738	0.00266328
Nme1	-4.95109	0.00340753
Abce1	-4.94386	0.00167681
Sgpp1	-4.92258	0.00260152
Sqle	-4.82873	0.00682105
Glo1	-4.76769	0.0034533
Sirpb1b	-4.62324	0.00285263
Acss1	-4.58092	0.0152201
Idh2	-4.57709	0.00155147
Bcl11a	-4.54405	0.0126847
Gm8221	-4.5369	0.000424934
Fli1	-4.53283	0.00158618
Pdia5	-4.50995	0.000424934
Gm19634	-4.46597	0.0134619
Lrmp	-4.46045	0.0039727
Tipin	-4.44897	0.00498815
Lbh	-4.42712	0.00658278
Cd200r1	-4.34753	0.0109817

Table 7-2: Top Genes Down Regulated in Response to *Listeria* Vaccination in CD8α^+ Dendritic Cells

Using Partek and Spotfire analysis software, transcript expression level was calculated as fold change vs. Vaccinia vaccinated CD8α^+ dendritic cell expression levels.

CHAPTER VIII

CONCLUSIONS AND FUTURE DIRECTIONS

The generation of potent, specific, anti-cancer immunity has been an aspirational goal of immunologists and clinicians alike. Toward this goal, significant progress has been made in understanding of the requirements for the generation and maintenance of the immune response. DNA vaccines, cell based vaccines, and live-attenuated vaccines all represent different methods of generating a new immune response, while checkpoint blockade, epigenetic therapy, and co-stimulatory signaling pathways all represent potential methods to refocus and reinforce a response already in progress. In pursuit of this goal, we have examined the anti-cancer and immunological effects of two, unique, live-attenuated anti-cancer vaccine vectors; *Listeria Monocytogenes* and Vaccinia Virus. *Listeria* based vaccinations resulted in superior control of tumor growth in multiple models, including multiple cancer lines as well as subcutaneous and metastatic models of disease. The anti-tumor effect of both vaccinations was dependent on the generation of an adaptive immune response, as shown by the lack of generated protection in $RAG2^{-/-}$ animals. Furthermore, the $RAG2^{-/-}$ mice receiving live-attenuated Vaccinia Virus vaccination actually died earlier, likely from the inability to control the vaccination dose. This highlights some of the concerns with the use of live-attenuated vaccines, and the status of the patients' immune system should be carefully considered prior to clinical use. Furthermore, the generation of anti-tumor immunity for our *Listeria* based vaccination was dependent on the generation of a $CD8^+$ T cell response, unlike the anti-tumor immunity generated by Vaccinia.

Since both vaccinations did generate $CD8^+$ T cell responses, though *Listeria* based vaccines generated a larger population, we thought there must also be functional differences in the $CD8^+$ T cells being primed, and therefore set out to utilize an adoptive

transfer model to study the earliest phenotypes of the responding $CD8^+$ T cells. We found that *Listeria* based vaccinations resulted in early increases in the percentage of cells producing single as well as multiple cytokines. $CD8^+$ T cells responding to *Listeria*-OVA produced more IFNγ, more TNFα, and more Granzyme B, all suggesting the potential for increased cytolytic activity on a per cell basis. However, $CD8^+$ T cells responding to Vaccinia-OVA produced more IL-2, which may represent the viral nature of this vector and therefore, the necessity for both a $CD4^+$ T cell and B cell response to be generated. These data were some of the first data we found supporting the hypothesis that these two vaccines were resulting in different programming of the resultant $CD8^+$ T cell population. *Listeria* resulted in increased expression of not all cytokines, but of certain, cytolytic cytokines. In terms of transcription factors, while Vaccinia vaccination did increase expression of the activating $CD8^+$ T cell transcription factors, it also resulted in up regulation of Egr2, a protein known in the $CD4^+$ T cell literature to be associated with anergy and a lack of function. This was unlike the response to *Listeria* vaccination, where there was little Egr2 expression, and increased Tbet and Eomesedermin expression. The role of Egr2 in $CD8^+$ T cell biology is not fully understood, and is a potential area for further exploration, as Egr2 expression may decrease overall cytolytic function in $CD8^+$ T cells as well.

The most pronounced difference between the $CD8^+$ T cell responses generated by these vaccines was the expression, or lack thereof, or the checkpoint proteins PD-1 and LAG-3. Recent publications from our lab, as well as others, have highlighted the roles of PD-1 and LAG-3 in the maintenance of peripheral tolerance as well as tumor tolerance. Furthermore, it is also known that blockade of PD-1 or LAG-3 can result in recovery of

function, increased cytokine production, and increased expansion not only in self-tolerance and tumor models, but also in models of chronic and acute infection. Classically, it is thought that the expression of checkpoint proteins during $CD8^+$ T cell activation is a requirement or side effect of the activation. However, in these studies, we found that PD-1 and/or LAG-3 expression is not a requirement of activation, but instead part of a specific program that can be prevented in the proper circumstances. Indeed, while the $CD8^+$ T cells responding to Vaccinia vaccination did up regulate PD-1 and LAG-3, $CD8^+$ T cells responding to *Listeria* vaccination did not express either of these checkpoints. The lack of PD-1 expression was further verified by comparison of PD-1 $^{-/-}$ OT-I $CD8^+$ T cells to wildtype OT-I $CD8^+$ T cells vaccinated by *Listeria*. Even when compared to genetic knockout animals, wildtype OT-I $CD8^+$ T cells expressed negligible amounts of PD-1 when responding to *Listeria* vaccination. Decreased expression of these two checkpoint proteins was not simply a function of the adoptive transfer model, as both a different adoptive transfer model (B10.D2 background) and examination of the endogenously generated tetramer positive cells showed similar results to these two vaccinations. Surprisingly, we found that *Listeria* and Vaccinia vaccines showed equivalent anti-tumor immunity when utilized in a PD-1 knockout host, suggesting that PD-1 expression is the molecular distinction that creates the functional difference for these two vaccines. Clinically, these data support the combination of checkpoint blockade therapy with vaccine strategies, as expression of PD-1 during the vaccination clearly affects the efficacy of the generated immune response. Furthermore, since many vaccines are known to cause PD-1 and LAG-3 expression during their priming phase, it is important to block these checkpoint proteins to achieve maximum efficacy. However,

these data are the first to demonstrate that some vaccines, such as this attenuated form of *Listeria Monocytogenes*,may activate a program in $CD8\alpha^+$ dendritic cells that can suppress up regulation of these checkpoint proteins, or at least minimize their expression. This is not to say that no checkpoint proteins are expressed, and further research will be necessary to examine other potential regulatory signaling molecules that may be compensating for PD-1 and LAG-3 in this system.

Finally, the role of the antigen presenting cells utilized by a vaccine strategy needs to be further explored when developing a vaccination platform. In these data, we have shown that both our attenuated *Listeria Monocytogenes* and Vaccinia Virus utilize one single population of dendritic cells, despite the large number of subpopulations capable of presenting antigen to $CD8^+$ T cells. $CD8\alpha^+$ dendritic cells are a subpopulation of the conventional dendritic cell lineage, and have been shown to be particularly powerful generators of $CD8^+$ T cell responses. While previous research has shown that the presence of $CD8\alpha^+$ dendritic cells is required for entrance of *Listeria* into the splenic architecture, it has never been clearly demonstrated that these dendritic cells are uniquely and specifically responsible for the generation of a $CD8^+$ T cell response to *Listeria Monocytogenes*. In these studies, we have shown that $CD8\alpha^+$ dendritic cells are the main population of dendritic cells responsible for priming *in vivo* responses to *Listeria Monocytogenes* based vaccinations, and that these cells alone are sufficient to drive $CD8^+$ T cell activation without the up regulation of PD-1 expression. The understanding of the targeted dendritic cell subset is important for the design of combination experiments. For example, early data describing the effects of GM-CSF from B16-GVAX in preclinical models has suggested that this vaccine expands out $CD8\alpha^-$ dendritic cells. When

combining a cell based vaccine with *Listeria*, it therefore may be more beneficial to utilize a therapy that expands $CD8\alpha^+$ dendritic cells, such as FVAX where the secreted soluble factor is the $CD8\alpha^+$ dendritic cell expanding factor Flt-3.

However, despite the utilization of the same subpopulation of dendritic cells, the immune responses generated by *Listeria Monocytogenes* and Vaccinia Virus are clearly different, both in the phenotypes of the $CD8^+$ T cells generated, as well as the general anti-tumor immunity. Therefore, we wanted to examine whether the differences in these vaccines would be reflected as early in the immune response as the antigen presenting cells. Surprisingly, after only twenty four hours, the transcriptional profiles of the $CD8\alpha^+$ dendritic cells responding to our two vaccinations were strikingly different. *Listeria Monocytogenes* vaccination resulted in the significant up regulation of transcripts known to be related to dendritic cell maturation, such as CISH, CD80, CD86, CD70, and the components of RIG-I signaling. Furthermore, *Listeria* vaccination also resulting in the down regulation of several signaling proteins, including RGS18, which is a GTPase activating protein, turning off G protein coupled signaling. Though the role of RGS18 in dendritic cells is relatively unknown, the down regulation of this transcript correlated with the up regulation of several known G protein coupled signaling pathways, suggesting a potential cascade effect that is involved in programming the dendritic cell to respond to certain stimuli, such as *Listeria Monocytogenes*. Nevertheless, the control of PD-1 expression on the $CD8^+$ T cells responding to either *Listeria* or Vaccinia does not seem to be dependent on soluble factors. This is different than what has been seen by other groups, where IL-10 or TGFβ have been found to be important regulators of PD-1 signaling. However, while those soluble factors have been found to increase PD-1

expression, no soluble factors to date have been shown to prevent up regulation of PD-1. The up regulation of this PD-1 modulating signaling pathway is independent of either MyD88 or STING signaling, suggesting that it is relatively specific, and not a feature of general dendritic cell maturation in response to pathogens. Furthermore, this pathway is only stimulated by *Listeria* that is incapable of leaving the cell it originally infects. Therefore, we propose a model whereby *Listeria* vaccination enters $CD8\alpha^+$ dendritic cells, escapes the endosome, and is stranded within the cytoplasm of these cells. From there, the *Listeria* stimulates a host innate sensing pathway that is independent of MyD88 or STING, and results ultimately in the up regulation of a protein or proteins that are responsible for programming the responding $CD8^+$ T cells to activate and expand without the up regulation of PD-1. *Listeria* also stimulates a MyD88 dependent pathway that is responsible for managing the level of LAG-3 expression on the responding $CD8^+$ T cells. Together, these data show that different innate pathways are responsible for the expression of different checkpoint proteins on the resultant, adaptive immune cells. These data also question the requirement for checkpoint protein expression during $CD8^+$ T cell activation, and demonstrates instead that checkpoints are individually regulated, and that their expression is not completely overlapping. Instead, the expression of each checkpoint may be individually mandated by the programming dendritic cell, and certain combinations may suggest certain signaling pathways involved or excluded during those cells activation or reactivation.

In the future, studies identifying the specific pathways and sensors responsible for the regulation of checkpoint proteins on $CD8^+$ T cells during activation will be of the utmost importance. Viral vectors could be potentially modified to generate a stronger

$CD8^+$ CTL response by intentionally signaling via pathways generally reserved for bacterial pathogens, such as *Listeria Monocytogenes*. Furthermore, the protein, or proteins involved in the modulation of PD-1 expression on CD8+ T cells needs to be identified and tested. Inclusion of this stimulatory protein in cancer therapies as well as during vaccine regimens has the potential to further expand the effectiveness of immunological clinical interventions. Knowledge of these pathways will allow for potential clinical interventions, by which the generation of an immune response could be tailored towards the specific type of immunity that is necessary, not only for cancer vaccines, but also for protective vaccinations in general.

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CURRICULUM VITAE FOR Ph.D. CANDIDATES

The Johns Hopkins University School of Medicine

Christopher J. Nirschl March 26, 14

Educational History

Ph.D. Expected	2014	Immunology Mentor: Charles G. Drake M.D., Ph.D	Johns Hopkins School of Medicine
B.S.	2009	Biology Mentor: Keith Kozminski Ph.D	University of Virginia
B.A.	2009	Chemistry Mentor: Keith Kozminski Ph.D	University of Virginia

Other Professional Experience

Undergraduate Researcher	2008-2009	Lab of Dr. Jay Hirsh, University of Virginia
Research Rotation	2009	Lab of Dr. Noel Rose, Johns Hopkins
Research Rotation	2009	Lab of Dr. Charles Drake, Johns Hopkins
Research Rotation	2010	Lab of Dr. T.C. Wu, Johns Hopkins

Academic and Other Honors

September 2011	Best Poster Presentation	JHU Immunology Program
Sept 2005- May 2009	Dean's List	UVA College of Arts and Sciences
May 2005	Eagle Scout	Boy Scouts of America, T913
May 2004	Bausch and Lomb Honoaray Science Award	Bausch and Lomb Inc

Peer Reviewed Publications

Durham, N.M., **Nirschl, C.J.**, Jackson, C., Elia, J., Ceccato, C., Anders, R., Drake, C.G. (In Submission). Lymphocyte Activation Gene (LAG-3) Modulates the Ability of CD4 T -cells to be Suppresed *in vivo*. *PLoS ONE*.

McAllister, F., Bailey, J., Alsina, J., **Nirschl, C.J**., Roeser, J., Lankapali, R., Zhang, H., Jaffee, E., Drake, C.G., Maitra, A., Kolls, J., Sears, C., Pardoll, D., Leach, S. (Accepted). Oncogenic Kras Activates IL-17+ Hematopoietic-to-Epithelial Signaling Axis in Preinvasive Pancreate Neoplasia. *Cancer Cell.*

Ellsworth, S., Balmanoukian, A., Kos, F., **Nirschl, C. J**., Nirschl, T. R., Grossman, S. A., Drake, C. G. (2014). Sustained CD4+ T cell-driven lymphopenia without a compensatory IL-7/IL-15 response among high-grade glioma patients treated with radiation and temozolomide. *OncoImmunology*, *2*(12), e27357.

Nirschl, C. J., & Drake, C. G. (2013). Molecular pathways: coexpression of immune checkpoint molecules: signaling pathways and implications for cancer immunotherapy. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *19*(18), 4917–24. doi:10.1158/1078-0432.CCR-12-1972

Woo, S.-R., Turnis, M. E., Goldberg, M. V, Bankoti, J., Selby, M., **Nirschl, C. J**., … Vignali, D. A. A. (2012). Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Research*, *72*(4), 917–27. doi:10.1158/0008-5472.CAN-11-1620

Zabransky, D. J., **Nirschl, C. J.**, Durham, N. M., Park, B. V, Ceccato, C. M., Bruno, T. C., … Drake, C. G. (2012). Phenotypic and functional properties of Helios+ regulatory T cells. *PloS One*, *7*(3), e34547. doi:10.1371/journal.pone.0034547

Published Symposium Presentations and Abstracts

Nirschl, C.J., Ceccato, C., Alme, A., Francica, B., Drake, C.G.(2013) Expression of Neuropilin-1 On in vivo Induced Regulatory T Cells. Society for the Immunotherapy of Cancer: 2013 Annual Meeting, Bethesda, MD, November 9, 2013.

Francica, B., **Nirschl, C.J.**, Drake, C.G.(2013) EGR2 and EGR3 as Effectors of Tolerance in Self Peptide Tolerized CD8+ Lymphocytes. Cancer Research Institute International Cancer Immunotherapy Symposium Series: 21st Annual Meeting, New York, New York, September 30, 2013.

Ceccato, C., **Nirschl, C.J**., Durham, N., Jackson, C., Alme, A., Drake, C.G.(2013) Tc17 Plasticity is Determined By the Inflammatory Milieu. Cancer Research Institute International Cancer Immunotherapy Symposium Series: 21st Annual Meeting, New York, New York, September 30, 2013.

Martin, A., **Nirschl, C.J.**, Polancyzk, M., Cohen, L., Pardoll, D., Drake, C.G., Lim, M. (2013). Myc Amplification Status Influences Tumor Immune Evasion in Medulloblastoma. Pediatric Neuro-Oncology Basic and Translational Research Conference: Society for Neuro-Oncology Bi Annual Meeting, Fort Lauderdale, FL, May 16, 2013.

Ceccato, C., Jackson, C., **Nirschl, C.J.**, Durham, N., Alme, A., Lim, M., Drake, C.G. (2013) Combining Radiotherapy and Immunotherapy in the Treatment of CNS Cancer. American Association of Immunologists: 99th Annual Meeting, Honolulu, Hawaii, May 3, 2013.

Boikos, S., **Nirschl, C.J.**, Martin, A., Alme, A., Harris, T., Drake, C.G.(2013) Prostate Cancer Cells Up-Regulate PD-L1 in Repsonse to Pro-Inflammatory Cytokines. American Association of Cancer Research: 104th Annual Meeting, Washington, DC, April 6, 2013.

McAllister, F., Bailey, J., Alsina, J., **Nirschl, C.J.**, Roeser, J., Blake, D., Lankapali, R., Sears, C., Jaffee, E., Kolls, J., Drake, C.G., Pardoll, D., Leach, S. (2013) TH17 Cells and Early Pancreatic Tumorigenesis. ASCO: 2013 Gastrointestinal Cancers Symposium, San Francisco, CA, January 24, 2013.

Martin, A., **Nirschl, C.J**., Polyancyyk, M., Cohen, K.J., Pardoll, D., Drake, C.G., Lim, M. Oncogenic Drivers of Medullablastoma May Determine a Novel Phenotype. International Society of Pediatric Neuro-Oncology: 17th Annual Meeting, Washington, DC, October 14, 2012.

Ceccato, C., Jackson, C., **Nirschl, C.J.**, Durham, N., Alme, A., Lim, M., Drake, C.G. (2012) Combining Radiotherapy and Immunotherapy in the Treatment of CNS Cancer. Cancer Research Institute Interanationional Cancer Immuotherapy Symposium Series: 20th Annual Meeting, New York, New York, October 1, 2012

McAllister, F., Bailey, J., Alsina, J., **Nirschl, C.J.,** Lankapalli, R., Roeser, J., Jaffee, E., Sears, C., Kolls, J., Drake, C.G., Pardoll, D., Leach, S. (2012) TH17 Cells in Early Pancreatic Tumorigenesis. American Associated for Cancer Research: Pancreatic Cancer: Progress and Challenges, Lake Tahoe, Nevada, June 18, 2012.

Nirschl, C.J., Martin, A., Alme, A., Drake, C.G. (2012) Expression of Immune Checkpoint Ligands on Prostate Cancer Cells: Implications for Adaptive Immune Escape. Patrick C. Walsh Foundation Symposium, Bethesda, MD, February 21, 2012.

Nirschl, C.J., Goldberg, M., Durham, N., Cecatto, C., Netto, G., Chaux, A., Drake, C.G. (2011) PD-1 and LAG-3 Work Synergistically to Prevent Anti-Tumor Immunity. Cancer Research Institute International Cancer Immunotherapy Symposium Series: 19th Annual Meeting, New York, New York, October 2, 2011.

Ceccato, C., Durham, N., **Nirschl., C.J.**, Alme, A., Pan, X., Tam, A., Drake, C.G. (2011) IL-2 and IL-7 Drive IL-17 Secreting CD8 Cells (Tc17) Toward IFN-g Production In Vitro. Cancer Research Institute International Cancer Immunotherapy Symposium Series: 19th Annual Meeting, New York, New York, October 2, 2011.

Bruno, T., Durham, N., **Nirschl, C.J**., Ceccato, C., Getnet, D., Pardoll, D., Drake, C.G. (2011) The Role of PD-1 in Human CD8 Prostate Infiltrating Lymphocytes. Elucidating the Mechansims of CD8 T Cell Plasticity. American Association of Immunologists: 98th Annual Meeting, San Francisco, CA, May 13, 2011.

Ceccato, C., Durham, N., Bruno, T., **Nirschl, C.J.**, Tam, A., Drake, C.G. (2011) Elucidating the Mechansims of CD8 T Cell Plasticity. American Association of Immunologists: 98th Annual Meeting, San Francisco, CA, May 13, 2011.

Goldberg, M., Woo, S., Bankoti, J., Selby, M., **Nirschl, C.J.**, Bettini, M., Vogel, P., Grosso, J., Netto, G., Chauz, A., Smeltzer, M., Workman, C., Pardoll, D., Korman, A., Vignali, D., Drake, C.G. (2011) The Checkpoint Molecules LAG-3 and PD-1 Synergize to Maintain Tolerance to Tumors. American Association of Cancer Research: 102nd Annual Meeting, Orlando, FL, April 2, 2011.

Service and Leadership

2011 - 2012	Tutor - Cell Structure and Dynamics Johns Hopkins School of Medicine, Baltimore, MD
2012-Present	Teaching Assistant – Graduate Immunology Johns Hopkins School of Medicine, Baltimore MD